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# Development of Cell Based Reporter Assay to Measure PKC-Delta Promoter Activity

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LOYOLA UNIVERSITY CHICAGO

DEVELOPMENT OF  
CELL-BASED REPORTER ASSAY TO MEASURE  
PKC- $\delta$  PROMOTER ACTIVITY

A THESIS SUBMITTED TO  
THE FACULTY OF THE GRADUATE SCHOOL  
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BY

KUSHAL PRAJAPATI

CHICAGO, IL

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## TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	x
ABSTRACT	xiii
CHAPTER 1: BACKGROUND	
Skin Cancer and Squamous Cell Carcinoma	1
PKC- $\delta$ as a Tumor Suppressor in SCC	2
Correlation between PKC- $\delta$ Loss and Ha-Ras	3
PKC- $\delta$ Loss on Transcriptional Level	4
PKC- $\delta$ Gene Regulation Studies	7
High Throughput Screening	10
Validation of HTS	12
Objectives and Hypothesis	13
Specific Aims	13
CHAPTER 2: MATERIALS AND METHODS	
Cell Culture	15
Drugs and Compounds	15
DNA Plasmids	16
Transfection	16
Dual Luciferase Assay	16
Flow Cytometry	17
Single Luciferase Assay	17
Luminescence and Fluorescence Measurements in PHERAstar FS	18
Cell Viability Assays	19
QRT-PCR	19
Statistical Analysis	19
CHAPTER 3: RESULTS	
Aim 1: To develop a stably transfected PKC- $\delta$ promoter-reporter cell line, suitable for high throughput screening.	
PGL3-hPKC $\delta$ -4.4 Isolation and Verification	20
Sensitivity of HaCaT-Ras Cells to Different Drugs	22
Transfection, Sorting of Transfected cells, and Selection of Clones	23
Screening of Selected Clones	27
Aim 2: To identify positive control compounds which induce PKC- $\delta$ promoter activity and validation of PKC- $\delta$ reporter assay.	

PP2 induces PKC- $\delta$ Promoter Activity in HaCaT-Ras Cells	30
Optimization of PHERAstar FS	31
Higher Doses of PP2 Lead to Higher Induction in PKC- $\delta$ Reporter Activity	37
Combination of Bay 11-7085 and TPA induces PKC- $\delta$ Reporter Activity	38
Z Value Calculation	41
Treatment of HaCaT-Ras Cells with PP2 Reduces Number of Attached Cells	41
EGFP Fluorescence does not reflect Number of Attached HaCaT-Ras Cells	43
Viability of HaCaT-Ras cells decreases after PP2 Treatment	45
Effect of Shaking on Variability of Luminescence	47
PKC- $\delta$ mRNA levels in HaCaT-Ras Cells	56
Ras mRNA levels in HaCaT-Ras Cells	57
PKC- $\delta$ mRNA levels in HaCaT-Fyn Cells	58
 CHAPTER 4: DISCUSSION	 61
 REFERENCES	 67
 VITA	 73

## LIST OF TABLES

Table	Page
1. Inferences of Z Values in High-Throughput Screening	12
2. Sensitivity of HaCaT and HaCaT-Ras cells to various drugs	22



## LIST OF FIGURES

Figure	Page
1. PGL3-hPKC $\delta$ 4.4 Construction	6
2. PGL3-hPKC $\delta$ 4.4 Plasmid Map	7
3. Potential Transcription Factor Binding Sites on the human PKC- $\delta$ Promoter	9
4. Proposed pathway leading to PKC- $\delta$ transcriptional repression after Ras Activation	10
5. Identification of 9.2 kb long- pGL3-PKC- $\delta$ -4.4 plasmid	21
6. Schematic of transfection and sorting of cells followed by selection of clones for screening	25
7. Flow cytometric sorting of highly EGFP+ HaCaT and HaCaT-Ras cells	26
8. Single EGFP+ HaCaT-Ras cell in a 96 well plate	27
9. HaCaT-Ras Clone Screening Batch 1	28
10. HaCaT-Ras Clone Screening Batch 2	29
11. Dual luciferase assay on HaCaT-Ras cells after different drug treatments	31
12. Fluorescence measurement in PHERAstar FS	32
13. Luminescence measurement in PHERAstar FS	33
14. Luminescence measurement in PHERAstar FS over different time points	34
15. Orbital averaging in PHERAstar FS improves precision of fluorescence measurements	36

16.	PP2 Dose-Response Relationship	38
17.	Testing different compounds for their ability to induce PKC- $\delta$ promoter activity	39
18.	Testing different compounds for their ability to induce PKC- $\delta$ promoter activity 2	40
19.	Effect of PP2 on morphology and number of attached HaCaT-Ras cells	42
20.	Poor correlation between fluorescence read by PHERAstar FS and number of attached cells observed by for HaCaT-Ras cells treated with different drugs	44
21.	Cell viability measurements using Alamar Blue Assay	45
22.	Cell viability measurements using Cell-Titler Fluor Viability Assay	46
23.	Shaking reduces variability of luminescence read-outs in PHERAstar FS	48-49
24.	Effect of shaking on PP2 treated HaCaT-Ras cells	51-52
25.	Shaking plate right after addition of luciferase reagent is not beneficial to reduce variability	54-55
26.	PKC- $\delta$ mRNA levels are not reduced in HaCaT-Ras cell line	56
27.	Ras mRNA levels are reduced in HaCaT-Ras cell line	57
28.	PKC- $\delta$ mRNA levels in HaCaT-Fyn and MDA-MB-231	59
29.	Dual luciferase assay on MDA-MB-231 cells after different drug treatments	60

## LIST OF ABBREVIATIONS

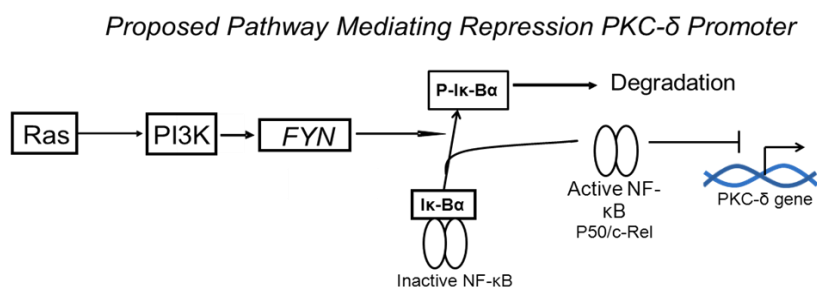
PKC	Protein Kinase C
PKC- $\delta$	Protein Kinase C- $\delta$
SCC	Squamous Cell Carcinoma
HTS	High Throughput Screening
QRT-PCR	Quantitative Real Time-Polymerase Chain Reaction
H.Ras	HaCaT-Ras
DNA	Deoxyribo Nucleic Acid
cDNA	complementary Deoxyribo Nucleic Acid
RNA	Ribo Nucleic Acid
mRNA	messenger Ribo Nucleic Acid
EGFP	Enhanced Green Fluorescent Protein
BCC	Basal Cell Carcinoma
DAG	Diacyl Glycerol

UV	Ultra Violet
GTP	Guanosine Tri Phosphate
EGF	Epidermal Growth Factor
TGF- $\alpha$	Transforming Growth Factor- $\alpha$
EGFR	Epidermal Growth Factor Receptor
kb	kilo bases
NF- $\kappa$ B	Nuclear Factor Kappa-light-chain enhancer of activated B cells
TNF- $\alpha$	Tumor Necrosis Factor- $\alpha$
AP-1	Activator Protein-1
ChIP	Chromatin Immuno Precipitation
PI3	Phospho-Inositide 3
PI3K	Phospho-Inositide 3 Kinase
AKR1C2	Aldo-Keto Reductase family 1, member C2
DMSO	Dimethyl Sulfoxide
DEPC	Di-Ethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
MEM	Minimum Essential Medium

PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin
nm	nano meter(s)
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
O.C	Optimal Concentration
o.m.p	over multiple passages
FACS	Fluorescence Activated Cell Sorting
PtdIns (4,5)P <sub>2</sub>	Phosphatidylinositol 4,5-Bisphosphate
PKC-d luc	pGL3-hPKC $\delta$ -4.4
PKC $\delta$ luc	pGL3-hPKC $\delta$ -4.4
FI	Fold Induction
CV	Co-efficient of Variation
CMV	Cytomegalovirus
LTR	Long Terminal Repeat

## ABSTRACT

Squamous cell carcinoma (SCC) is the second most common type of skin cancer in the United States with around 3.5 million cases diagnosed every year. Protein Kinase C (PKC) is a family of 9 serine/threonine kinases having distinct role in cell proliferation, differentiation, apoptosis and angiogenesis. One widely expressed isoform of PKCs, PKC- $\delta$  has been shown to act as a tumor suppressor in skin cancer by mediating cell



apoptosis. Re-expression of PKC- $\delta$  in human SCC cells induced apoptosis and suppressed tumorigenicity *in vitro*. PKC- $\delta$  expression is lost in 30% of human SCC tumors and is repressed in keratinocyte cell lines expressing activated HRAS. The Denning lab showed earlier that this loss of PKC- $\delta$  is at the transcriptional level and involves the Ras pathway shown below.

We hypothesize that compounds inhibiting this pathway will re-induce PKC- $\delta$  gene expression and will be effective therapeutic agents in SCC. In order to test the hypothesis, we proposed development of a high throughput, cell-based reporter assay on

Ras transformed keratinocyte cell line, HaCaT-Ras to measure PKC- $\delta$  promoter activity and screen compounds having potential to induce PKC- $\delta$  promoter activity.

In this study, we utilized a PKC- $\delta$  promoter reporter plasmid (pGL3-hPKC $\delta$ -4.4) generated earlier in our lab to assess PKC- $\delta$  promoter activity, in which 4.4 kb region of human PKC- $\delta$  promoter was inserted next to luciferase reporter gene. The first goal of this project was to develop a HaCaT-Ras cell line stably transfected with PKC- $\delta$  reporter plasmid. To do this, we co-transfected HaCaT-Ras cells with PKC- $\delta$  reporter and EGFP plasmids, and sorted EGFP positive cells by flow cytometry to select clones which were stably transfected with EGFP. In the next step, clone screening, we identified one clone having stable PKC- $\delta$  reporter expression. Thus we were successful in generating a stable, PKC- $\delta$  promoter-reporter transfected HaCaT-Ras cell line.

The second aim of this study was to identify positive control compound(s) that give high induction in PKC- $\delta$  promoter activity to characterize and validate this assay. To pursue this aim, we tested 13 compounds belonging to different pharmacological classes either alone or in the combination for their ability to induce PKC- $\delta$  promoter activity on HaCaT-Ras cells transiently transfected with PKC- $\delta$  reporter. We identified src-family kinase inhibitor PP2 and the combination of NF- $\kappa$ B inhibitor Bay11-7085 and PKC activator TPA as reasonable positive control compounds for this assay. We proposed to perform this assay on microplate reader PHERAstar FS, and optimized the protocol on this instrument so that resulting high-throughput screening assay has higher Z value (statistical parameter directly related with quality of high-throughput screening) and convenience. After series of experiments which tested effects of various time-points of luminescence measurements, optic settings, and durations of shaking on quality of assay,

we were able to generate a protocol which offered less variability and accurate signal measurement on PHERAstar FS.

To conclude, in this project we generated a HaCaT-Ras cell line stably transfected with PKC- $\delta$  promoter-reporter plasmid (pGL3-hPKC $\delta$ -4.4), and a protocol for high throughput luciferase reporter assay in PHERAstar FS. Overall, these studies will be very helpful in future for developing a robust, high-throughput screening assay to screen large number of compounds which have potential to induce PKC- $\delta$  gene expression.



## CHAPTER 1

### BACKGROUND

#### **Squamous Cell Carcinoma**

Skin cancer is the most frequent form of cancer in the United States with around 3.5 million new cases diagnosed every year (1). There are two major types of skin cancers depending on their cells of origin in the skin tissue. In melanoma skin cancer, cancer originates in melanocytes, cells responsible for producing the pigment melanin. The other type of skin cancer is non-melanoma skin cancer, where cancer originates from keratinocytes in the skin. Non-melanoma skin cancer is again divided into two sub-classes namely basal cell carcinoma (BCC) and squamous cell carcinoma (SCC).

Squamous cell carcinoma (SCC) is the second most common (around 20% of non-melanoma skin cancers) type of skin cancer in the US (2, 3). As SCCs may be fatal if not treated properly, a safe and efficacious therapeutic strategy is required to make treatment better, and reduce its mortality rate. Currently primary treatment option for SCC includes surgery. However, it has adverse effects such as skin scarring, irritation, redness, loss of pigments, and is economically expensive. To avoid these clinical side-effects and make treatment more economically affordable to patients, a second treatment approach, chemotherapy may be used to treat SCC. However, applicability of this approach is limited by the fact that only a handful of topical agents such as 5-fluorouracil, and imiquimod are available to treat SCC today. Hence, more effective pharmacological

agents that alleviate and eliminate SCC are of crucial need to provide better treatment options to SCC patients.

### **PKC- $\delta$ as a tumor suppressor in SCC**

Protein Kinase C (PKC) is a family of 9 serine/threonine kinases having distinct role in cell proliferation, differentiation, apoptosis and angiogenesis (4, 5). PKCs reside in the cell cytosol in their inactive state. Activation of most PKCs takes place by their recruitment into the cell membrane and allosteric activation by diacylglycerol (DAG) (4, 5). Calcium enhances the process of activation of classical PKCs- $\alpha$ ,  $\beta$ , and  $\gamma$  (4, 5). When upstream mediator of the signal is a tyrosine kinase receptor, production of DAG in the cell membrane results from cleavage of phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5)P<sub>2</sub>) by phospholipase C- $\gamma$  (PLC- $\gamma$ ) (4, 5). In contrast when the signaling is conducted by G protein-coupled receptors (GPCRs), G $\alpha_q$  subunit interacts with PLC- $\beta$  which results in cleavage of PtdIns (4,5)P<sub>2</sub> and synthesis of DAG in the cell membrane (4, 5). Once activated, PKCs are phosphorylated on their kinase domains and phosphorylate their downstream substrates which can result in any of the different biological effects of PKCs including regulation of cell growth and differentiation.

One widely expressed isoform of PKCs, PKC- $\delta$ , induces growth arrest in several types of cells upon its activation (6, 7) and is shown to be pro-apoptotic in renal, neuronal and breast cell lines (8, 9, 10). Our lab has previously shown that PKC- $\delta$  is both necessary and sufficient for apoptotic cell death in human keratinocytes after exposure to UV light (11, 12, 13). PKC- $\delta$  is activated by caspase-3 mediated cleavage in response to apoptotic stimulus resulting in its constitutionally active catalytic fragment in human keratinocytes (11). This constitutionally active PKC- $\delta$  fragment in turn phosphorylates and

downregulates anti-apoptotic Mcl-1 protein in mitochondria, resulting in cytochrome C release and apoptosis (14). Over-expression of PKC- $\delta$  in keratinocytes by retroviral infection reduced their growth by inducing apoptosis *in vitro* (15).

A tumor suppressor gene is defined as a gene which prevents or inhibits formation of tumors, and its expression is often lost in the cancerous tissue. The Denning lab explored PKC- $\delta$ 's role as a tumor suppressor in squamous cell carcinoma and found that 30% of human SCC-tissue samples showed loss or reduction of PKC- $\delta$  protein (15). Also, transgenic mice over-expressing PKC- $\delta$  are resistant to chemically induced SCCs (16). Interestingly, re-expression of PKC- $\delta$  in HRAS transformed human keratinocytes grafted in nude mice dramatically inhibited tumor growth *in vivo* (15). Taken together, it is clear that PKC- $\delta$  plays prominent role in eliminating pre-cancerous cells by mediating UV induced apoptosis in human keratinocytes, and has tumor suppressor properties in SCC. Thus increasing expression or activity in SCC might be a promising strategy to develop novel therapeutics for SCC.

### **Correlation between PKC- $\delta$ loss and Ha-Ras**

Ras is a family of small GTPase proteins found in human cells encoded by three genes named as HRAS, KRAS and NRAS. These GTPase proteins are activated through various external stimuli (e.g. mitogens such as EGF) and subsequently activate downstream effectors that control cell growth, differentiation and survival. Gene amplification or activating mutation in Ras gene leads to aberrant cell growth or differentiation, often leading to cancer (17, 18).

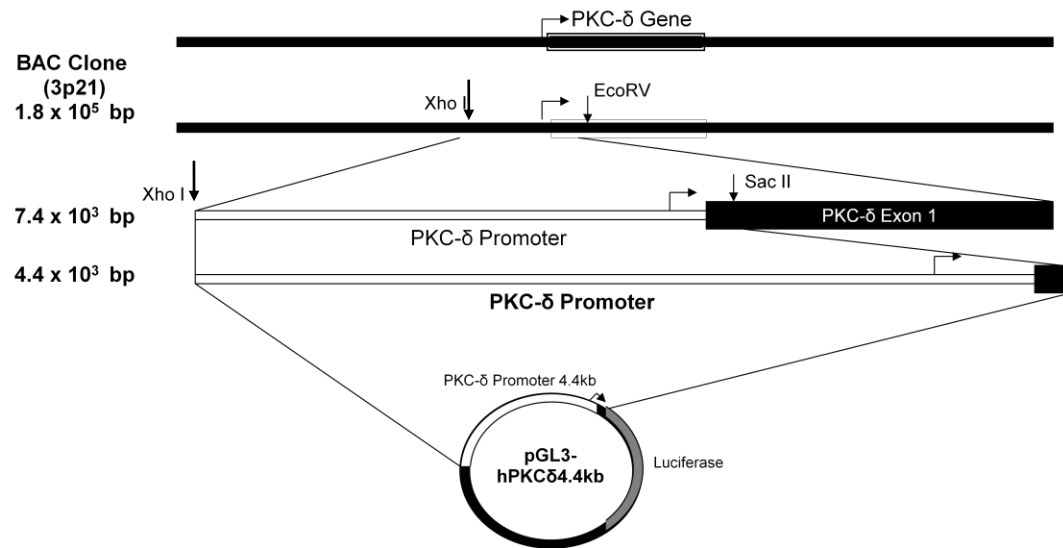
As in other human cancers, activating Ras mutations are found in SCCs, where around 50% of SCCs carry activating Ras mutations (19). As significant proportion of human

SCCs have active Ras mutations (19) as well as loss of PKC- $\delta$  (15), it is possible that these both events are correlated with each other in human SCCs. In Over 90% of chemically induced mouse tumors have been shown to carry activating Ha-Ras (a member of Ras family of proteins, encoded by HRAS gene) mutations (20). In the HaCaT immortalized human keratinocytes cell line, expression of activated Ha-Ras reduces PKC- $\delta$  protein and mRNA levels (21). It has also been demonstrated earlier that *in vitro* transduction of active Ras in keratinocytes isolated from mice, highly induces mRNA expression of several EGFR ligands such as TGF- $\alpha$ , heparin binding EGF like factor, betacellulin (22). This phenomenon results in generation of autocrine loop by active Ras, where high levels of EGFR ligands lead to over-activation of EGFR in Ras transformed keratinocytes. Furthermore, majority of human SCC-tissue samples which showed reduced PKC- $\delta$  levels also stained positive for phospho-EGFR (15) suggesting activation of Ras in these tumors (15). Phospho-EGFR was not detected in normal human skin (15). Thus we propose that it is activating Ha-Ras mutation that confers reduction in PKC- $\delta$  protein expression.

### **PKC- $\delta$ loss on transcriptional level**

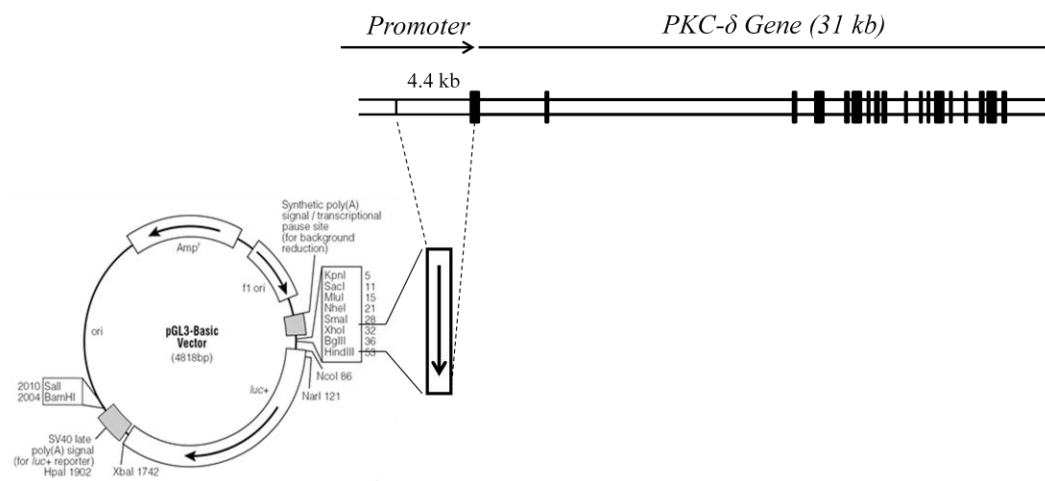
The Denning lab published in 2010 that loss of PKC- $\delta$  expression in human SCC is on transcriptional level (23). They selected 14 human SCC-tissue samples which showed loss of PKC- $\delta$  expression in immunohistochemistry, and all of these samples expressed low PKC- $\delta$  mRNA levels, when measured by qRT-PCR (23). Also in gene-deletion analysis using qPCR, 8 out of 9 SCC tumors had intact PKC- $\delta$  gene, supporting transcriptional repression as a major process contributing to PKC- $\delta$  loss in SCC (23).

After establishing a relationship between activating Ras mutations and PKC- $\delta$  loss earlier, our lab extended studies on Ras transformed HaCaT cells *in vitro*. Ras transformed HaCaT cells, named as HaCaT-Ras had lower endogenous PKC- $\delta$  mRNA levels compared to HaCaT cells (23). To assess PKC- $\delta$  promoter activity, the promoter region of human PKC- $\delta$  gene from a BAC clone (RPCI-11-82B23, BACPAC Resources, Children's Hospital Oakland Research Institute) was sub-cloned into the firefly luciferase reporter construct pGL3-Basic vector (Promega) to generate pGL3-hPKC $\delta$ -4.4 plasmid (23). As shown in the Figure 1, a 7.4 kb region of PKC- $\delta$  gene was excised from BAC clone using XhoI and EcoRV enzymes in first step. Then, 4.4 kb region of PKC- $\delta$  promoter was excised using XhoI and SacII enzymes and sub-cloned into XhoI and HindIII restriction sites of pGL3-basic vector. Resulting pGL3-hPKC $\delta$ -4.4 construct was then used to measure PKC- $\delta$  promoter activity in different cell lines. When this construct was transfected into HaCaT-Ras cells, they showed significantly less PKC- $\delta$  promoter activity compared to HaCaT cells (23). This result confirmed that Ras mediated PKC- $\delta$  transcriptional loss in keratinocytes is due to reduction in PKC- $\delta$  promoter activity.



**Figure 1: pGL3-hPKCδ4.4 Construction:** 7.4 kb fragment of PKC-δ gene of was excised from 180 kb-BAC clone, followed by sub cloning 4.4 kb region of PKC-δ promoter into pGL3 basic vector.

Figure 2 illustrates restriction-site map of pGL3-hPKCδ-4.4.



**Figure 2: pGL3-hPKC $\delta$ 4.4 Plasmid Map:** 4.4 kb promoter region of PKC- $\delta$  gene was sub-cloned into pGL3 basic vector between restriction sites XhoI and HindIII (17). Total length of pGL3-hPKC $\delta$ 4.4 was 9.2 kb. Diagram of pGL3-Basic Vector is a copyright of Promega Corporation.

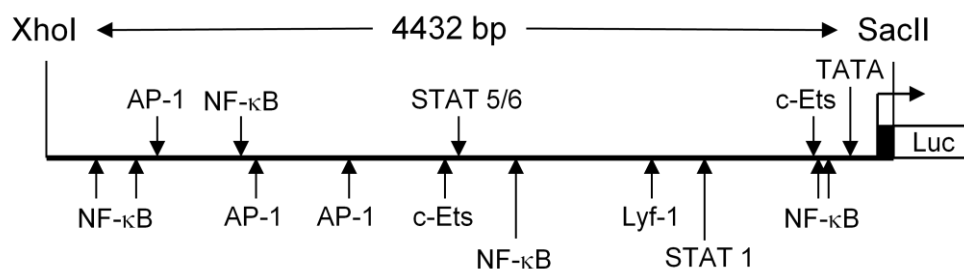
### PKC- $\delta$ gene regulation studies

Although functions of PKC- $\delta$  have been studied widely, few studies have been done to understand its gene regulation. The 31 kb long human PKC- $\delta$  gene is located at 3p21.31 region of the human chromosome (24). It comprises of 18 exons. In 2003, Suh *et al.* studied a 1.7 kb promoter region of murine PKC- $\delta$  gene, and showed that NF- $\kappa$ B increases PKC- $\delta$  promoter activity, resulting in increase in its expression (24). When mouse keratinocytes were transfected with murine PKC- $\delta$  promoter and treated with TNF- $\alpha$ , an activator of NF- $\kappa$ B, they showed higher reporter activity compared to untreated keratinocytes (24). This increase in reporter activity by TNF- $\alpha$  was abrogated by infecting keratinocytes with superrepressor I $\kappa$ B, a negative regulator of NF- $\kappa$ B (24). Liu and co-workers in 2006 showed that RelA, a major transactivating subunit of NF- $\kappa$ B positively regulates PKC- $\delta$  expression in mouse fibroblasts (25). This positive regulation was a result of increase in PKC- $\delta$  promoter activity mediated by interaction of RelA with

two NF- $\kappa$ B binding sites identified in the same studies at -83 to -74 and -52 to -43 sites in -309 to -1 region of human PKC- $\delta$  promoter (25). Other studies done on PKC- $\delta$  gene regulation in past demonstrated that molecules p63, p73 in keratinocytes and SP1 in skeletal muscle cells also play role in positively regulating PKC- $\delta$  promoter activity (26, 27).

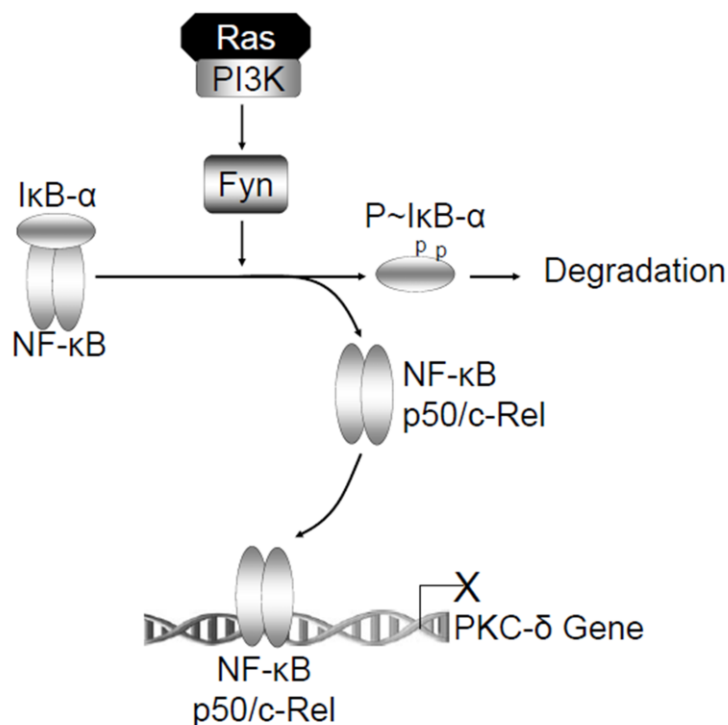
A former Ph.D. student of the Denning lab, Vipin Yadav attempted to dissect the signaling pathway downstream of activated Ha-Ras that leads to transcriptional repression of PKC- $\delta$  promoter activity. In his study, TFSearch analysis identified many potential transcription factor binding sites such as NF- $\kappa$ B, c-Ets, AP-1 in 4.4kb long human PKC- $\delta$  promoter (Figure 3). Site directed mutagenesis of NF- $\kappa$ B binding motif at -311 site in PKC- $\delta$  promoter resulted in 20 fold increase in PKC- $\delta$  promoter activity in HaCaT-Ras cells. Further, ChIP analysis using PCR primers that corresponded to NF- $\kappa$ B binding sites at -311 and -301 on PKC- $\delta$  promoter revealed that p50 and c-Rel subunits of NF- $\kappa$ B are specifically recruited to PKC- $\delta$  promoter in HaCaT-Ras cells. These results suggested that NF- $\kappa$ B plays prominent role in Ras mediated repression of the PKC- $\delta$  promoter. The data he generated further suggests that activated Ras activates PI3K, which activates Fyn (Src-family kinase), which subsequently phosphorylates I $\kappa$ B- $\alpha$ , a negative regulator of NF- $\kappa$ B (Yadav, Denning, Personal communication, Figure 4). This leads to degradation of I $\kappa$ B- $\alpha$  and activation of NF- $\kappa$ B, which finally represses PKC- $\delta$  promoter activity (Yadav, Denning, Personal communication, Figure 4). This proposed mechanism of active Ha-Ras leading to low PKC- $\delta$  promoter activity (Yadav, Denning, Personal communication, Figure 4) might be useful in devising therapeutic strategies to increase PKC- $\delta$  promoter activity in human keratinocyte cell line.





**Figure 3: Potential Transcription Factor Binding Sites on the Human PKC- $\delta$  Promoter:** In schematic depicting potential transcription factor binding sites on 4.4 kb PKC- $\delta$  promoter region, please note the functional NF- $\kappa$ B binding site - 311 (second arrow pointing upward from the right side) on promoter.

Yadav V, Denning MF, Personal communication



**Figure 4: Proposed pathway leading to PKC- $\delta$  transcriptional repression after Ras activation:** Activated Ras activates PI3K, which further activates src-family kinase Fyn. Activated Fyn in turn phosphorylates and degrades I $\kappa$ B- $\alpha$  which activates NF- $\kappa$ B comprising p50 and c-Rel subunits. NF- $\kappa$ B finally translocates into nucleus and repressed PKC- $\delta$  promoter.

### High Throughput Screening

High throughput screening (HTS) is a process of drug-discovery in which large numbers of molecules belonging to the pharmacological classes of interest are tested for their ability to produce measurable biological or chemical change in the system in highly accurate, sensitive, convenient, automated, as well as time and cost-efficient manner. Chemical compound libraries might contain 100,000 to as many as 1 million molecules,

high throughput screening of which is conducted mainly in 96, 384 or 1536 well plate to allow miniaturization and automation. It also often involves robotics and automatic liquid handling systems. In screening, compounds which have desired biological or chemical effect of quantum above the set threshold are called 'hits'. These hits are subjected to additional biochemical, pharmacological and clinical tests in next steps to find out the best compound having potential to become a therapeutic agent.

HTS majorly involves two types of assays, namely biochemical and cell-based assays (28). Bio-chemical assays include isolation of biological or chemical target, and direct measurement of effect of drug candidates on target *in vitro* in environment suitable for biochemical measurements (29-35). Cell-based assays, which are performed on cells grown *in vitro* (36-41), are increasingly preferred over biochemical assays for HTS due to their similarity to physiological environment and larger scope of studies extending beyond just one target to multiple targets and whole signaling pathways (28). In addition to these two conventional approaches, many whole organism-based screening assays have been developed (42-52) in attempt to make HTS as physiologically relevant as possible.

Cell-based assays represent at least half of all the HTS assays performed today (53). Major subtypes of cell-based assays are second messenger, reporter gene and cell proliferation or toxicity assays (53). Cell-based reporter gene assays are widely used to measure transcriptional or promoter activity of gene of interest. There have been many studies where cell-based reporter assays are successfully developed in high throughput format (38, 39, 54-57). Recently a group of researchers in Switzerland developed a HaCaT keratinocyte-based, high throughput luciferase reporter assay to test compounds

for their ability to sensitize the skin by inducing promoter of human AKR1C2 gene (58). In this study, HaCaT cell line stably transfected with pGL3 basic vector carrying antioxidant response elements (AREs) of human AKR1C2 gene was created using drug selection approach (58). This cell line was then used to screen numerous compounds and assess the statistical parameters of the assay (58).

### Validation of HTS

After HTS assay has been designed, it is validated using statistical parameters. Zhang et al has very well defined a parameter to assess usefulness of assay for high throughput screening known as Z-factor (59). Z-factor is defined as follows (59):

$$Z = 1 - \frac{3 \text{ Standard Deviation of Positive Control} + 3 \text{ Standard Deviation of Negative Control}}{|\text{Mean of Positive Control} - \text{Mean of Negative Control}|}$$

Following are the interpretations of Z values for HTS (59):

**Table 1: Inferences of Z values in High-Throughput Screening (59)**

<b>Z value</b>	<b>Inference for screening</b>
1	An ideal assay.
$0.5 \leq Z < 1$	An excellent assay
$0 < Z < 0.5$	A doable assay
0	A yes/no type assay
$< 0$	Screening essentially impossible

### **Objective and hypothesis**

Our objective was to develop a stable cell-based reporter assay that could accurately detect relative PKC- $\delta$  promoter activity so that high throughput screening of compound libraries could be performed to identify compounds that induce PKC- $\delta$  transcription. The identified compounds (also referred to as 'hits') could be suitable candidates for novel drug development project for SCC, as even small increase in apoptosis can cause relatively large growth inhibition in regressing skin tumors (60). We proposed following hypothesis for this project.

Compounds belonging to specific classes such as NF- $\kappa$ B inhibitors, or src-family kinase inhibitors, induce PKC- $\delta$  gene expression in stably transfected, PKC- $\delta$  reporter-human keratinocytes cell line which is Ras-transformed.

We identify following specific aims to test this hypothesis.

### **Specific Aims**

**Aim 1: To develop a stably transfected PKC- $\delta$  promoter-reporter cell line, suitable for high throughput screening.**

Aim of developing a stable PKC- $\delta$  reporter cell line, suitable for high throughput screening forms the backbone of this project, as this cell line could be used in developing assay as well screening of compounds.

This aim has been divided into following sub-aims for convenience.

**Sub-Aim 1A: Transfection and sub-subsequent selection/sorting of transfected cells followed by selection of clones.**

**Sub-Aim 1B: Screening of selected clones.**

**Aim 2: To identify positive control compounds which induce PKC- $\delta$  promoter activity and validation of PKC- $\delta$  reporter assay.**

This aim has been divided into following sub-aims for convenience.

**Sub-Aim 2A: To identify positive control compounds which induce PKC- $\delta$  promoter activity.**

It is very important for this project to identify in prior some compounds which induce PKC- $\delta$  gene expression to use them as positive controls for screening libraries of compounds.

**Sub-Aim 2B: Validation of PKC- $\delta$  reporter assay.**

Validation of assay i.e. all experimental conditions such as clone, positive control, plating density is highly important step before going to screening stage. It allows researcher to assess whether the assay is applicable to high throughput screening or needs further optimization.

**Sub-Aim 2C: Investigating Ras and PKC- $\delta$  expression in HaCaT-Ras and other cell lines.**

## CHAPTER 2

### MATERIALS AND METHODS

#### **Cell Culture**

Both the HaCaT and HaCaT-Ras cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin Streptomycin (Pen Strep). MDA-MB-231 cells were cultured in Improved Minimum Essential Medium (IMEM, VWR, Radnor, PA) supplemented with 5% FBS, 1% L-glutamine, and 1% non essential amino acids. To enhance growth of single HaCaT and HaCaT-Ras cells plated in 96 well plates after flow-cytometric cell sorting, either medium was supplemented with 10 ng/mL of human EGF, or regular medium and conditioned medium (medium collected from plate of similar cells when cells reached around 80% confluency) were used in 1:1 proportion.

#### **Drugs and compounds**

Compounds used in this project were PP2 (Life Technologies, Carlsbad, CA), Dasatinib (LC Laboratories, Woburn, MA), LY294002 (Alexis Biochemicals, San Diego, CA), Bay 11-7085 (Santa Cruz Biotechnology, Dallas, TX), Bortezomib (Millennium Pharmaceuticals, Cambridge, MA), MG132, GDC-0941 (Chemietek, Indianapolis, IN), Wortmannin (Alexis Biochemicals, San Diego, CA), Vitamin D3 (Sigma, St Louis, MO), TNF- $\alpha$ , estrogen (Sigma, St Louis, MO), insulin and TPA (Alexis Biochemicals, San

Diego, CA). Stock solutions were made in either DMSO or DEPC-treated water depending on vehicle recommended by manufacturer for different compounds.

### **DNA Plasmid**

pGL3-hPKC $\delta$ -4.4 has been described previously (Figure 2, Reference 23). Renilla luciferase control pRL-CMV was purchased from Promega, Fitchburg, WI. EGFP plasmid pEGFP-C1 (Clontech, Mountain View, CA) was a gift from Dr. Clodia Osipo.

### **Transfection**

Transfections were performed using TransIT 2020 (Mirus, Madison, WI) or FuGENE 6 (Roche, Indianapolis, IN) by following manufacturer's protocol. Briefly, on the day of transfection media on the cells was removed and replaced with regular DMEM for TransIT 2020 or serum-free Opti-MEM (Life Technologies, Carlsbad, CA) for FuGENE 6. Recommended quantities of plasmid DNA and transfection reagent were mixed and incubated at room temperature for 30 minutes to allow complex formation, followed by addition of appropriate volume of this mixture into each well. Amounts of plasmid DNA transfected for different experiments are reported in their individual sections. MDA-MB-231 cells were transfected using Lipofectamine (Life Technologies, Carlsbad, CA) according to manufacturer's protocol.

### **Dual Luciferase Assay**

HaCaT or HaCaT-Ras cells were plated on 12 well plate at density of 150,000 cells or 200,000 cells per well respectively. 24 hours after plating, firefly luciferase reporter plasmid (pGL3-hPKC $\delta$ -4.4) and renilla luciferase control plasmid (pRL-CMV) were transfected into cells using TransIT 2020 or FuGENE 6 by methods described in Transfection section. In each well of a 12 well plate, 0.5  $\mu$ g of firefly and 0.05  $\mu$ g of



renilla luciferase plasmids were transfected. In experiments where cells were treated with drugs, drug treatments were started 24 hours after transfection. 48 hours after starting drug treatment, cells were lysed and firefly and renilla luciferase activities were measured using Dual Luciferase Reporter Assay (Promega, Fitchburg, WI) as per manufacturer's instructions in Zylux bench-top luminometer. In experiments where cells were not treated with drugs, 48 hours after transfection luciferase activities were measured.

### **Flow Cytometry**

Cells were washed with PBS- once and trypsinized. After trypsinization cells were suspended either in PBS with 1% BSA in PBS or DMEM with 10% FBS, followed by sorting of highly EGFP positive cells in BD FACSAria III (Becton Dickinson, Franklin Lakes, NJ). Each sorting was performed on single cells gated from a mixed population. Wherever necessary, sorted cells were directly plated in 96 well plate as single cell per well.

### **Single Luciferase Assay**

Cells were plated on p60 or 6 well-plate in a way that they were around 60% confluent the next day. 24 hours after plating, pGL3-hPKC $\delta$ -4.4 was transfected into cells using FuGENE 6. Roche recommended ratio of 3  $\mu$ L of FuGENE 6 for 1  $\mu$ g of pGL3-hPKC $\delta$ -4.4 was used for transfection. One day after transfection, HaCaT or HaCaT-Ras cells were plated in 96 well plate at a density of 20,000 or 50,000 cells per well respectively. Volume of DMEM in which cells were plated was 95  $\mu$ L per well. The next day, drug treatments were initiated on cells by adding 5  $\mu$ L of drug stock solutions per well, where concentrations of stock solutions were 20 times higher than the required concentrations. 48 hours after starting drug treatments, luminescence was measured in PHERAstar FS

microplate reader (BMG Labtech, Cary, NC) using Steady-Glo Luciferase Assay System (Promega, Fitchburg, WI) following Promega's protocol. For experiments where cells were not treated with drugs, luminescence was measured 48 hours after plating cells. Time point of luminescence measurement after addition of Steady-Glo luciferase assay reagent varied with different experiments.

### **Luminescence and Fluorescence Measurements in PHERAstar FS**

All measurements were performed on PHERAstar FS with the help of BMG Labtech's software and operating manual. For luminescence measurements, automatic injection function of PHERAstar FS was used to add luciferase assay reagent into 96 well plates. Speed of injection was chosen as 100  $\mu\text{L}/\text{second}$ . 10 minutes after adding reagent, luminescence was measured in a 'Plate Mode' using 'Bottom Optics'. Plate was read after enabling 6 mm of 'Orbital Averaging', a function which averages out multiple read-outs taken across a specific diameter instead of taking just one read-out from a single point. Delay was 0.52 seconds. Focal height adjustment was performed on whole plate every time before reading luminescence. In experiments where plates were shaken after injecting the reagent, more than one kinetic cycle were chosen depending on experimental conditions such as shaking time, number of measurements, otherwise plates were read using one kinetic cycle. Shaking was performed by choosing 'Additional Shaking' option in injection settings, where required speed and mode of shaking was chosen.

Fluorescence intensities were measured in 'End Point Mode', using appropriate excitation and emission module in PHERAstar FS. Before each measurement focal height and gain were adjusted for whole plate.

### **Cell Viability Assays**

For Alamar Blue assay (Life Technologies, Carlsbad, CA), 10  $\mu$ L of Alamar Blue reagent was added to 100  $\mu$ L of media in each well of 96 well plates. Then the plates were incubated at 37° C for approximately 2 hours. After incubation, fluorescence on plates was read using excitation/emission filter of 544/590 nm on POLARstar Omega (BMG Labtech, Cary, NC) as per manufacturer's instructions. To perform Cell Titer-Fluor Cell Viability Assay (Promega, Fitchburg, WI), manufacturer's protocol was followed.

### **qRT-PCR**

For qRT-PCR, total RNA was isolated from cells using TRIzol Reagent (Life Technologies, Carlsbad, CA) following manufacturer's protocol. Isolated RNA was converted to cDNA, and qRT-PCR was performed using SYBR Green real-time PCR kit (Life Technologies, Carlsbad, CA). GAPDH was a normalizing control for all the qRT-PCRs. The sequences of primers were 5'-CAGATTGTGCTAATGCGGGC-3' (Forward), 5'-TTTGCAATCCACGTCCTCCA-3' (Reverse) for PKC- $\delta$ , 5'-AGTCGCGCCTGTGAACG-3' (Forward), 5'-CGTCATCGCTCCTCAGGG-3' (Reverse) for Ha-Ras, 5'-GGCAGCCCTGTACGGGAGGT-3' (Forward), 5'-GCTCCACCTGCTCCAGCACC-3' (Reverse) for Fyn, and 5'-ACACTCAGCATCATCAAACCTCAA-3' (Forward), 5'-TTCAGTGATAGCATCACCATGTC-3' (Reverse) for GAPDH.

### **Statistical Analysis**

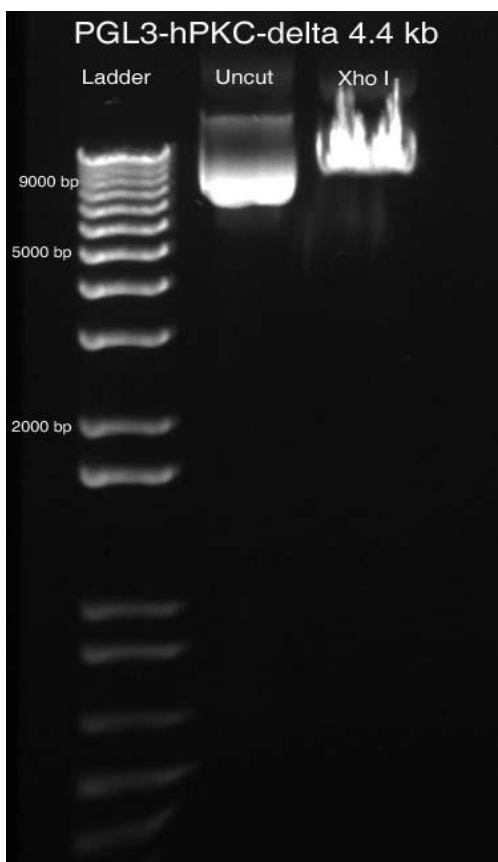
Student's T test was performed to calculate the P values and determine the statistical significance of the data. The difference observed between two experimental groups was considered statistically significant if the P value was less than 0.05.

## CHAPTER 3

### RESULTS

#### **pGL3-hPKC $\delta$ -4.4 isolation and verification**

To transfect PKC- $\delta$  promoter-reporter plasmid into cells, we isolated pGL3-hPKC $\delta$ -4.4, which is firefly luciferase reporter plasmid of 4.4 kb human PKC- $\delta$  promoter, from bacteria using QIAGEN Plasmid Midi kit according to manufacturer's protocol. The length of isolated plasmid was verified by linearizing it with restriction enzyme XhoI, and running it on 0.8% agarose gel. In Figure 5, length of a linearized plasmid is approximately 9 kb (lane 3), which closely resembles length of pGL3-hPKC $\delta$ -4.4 (9.2 kb, Figure 2).



**Figure 5: Identification of 9.2 kb long- pGL3-PKC- $\delta$ -4.4 plasmid:** 1  $\mu$ g of pGL3-PKC- $\delta$ -4.4 plasmid was run on 0.8% agarose gel either uncut or linearized after digesting with XhoI.

### Sensitivity of HaCaT-Ras cells to different drugs

In order to develop a stably transfected HaCaT and HaCaT-Ras cell lines using drug-selection approach, we sought to determine drugs to which both the cell lines were sensitive, and the optimal concentrations of drugs at which all the sensitive cells on the plates were killed. To determine this, we treated HaCaT and HaCaT-Ras cells with different concentrations of different drugs over different time durations. Results of these experiments are summarized in following table.

**Table 2: Sensitivity of HaCaT and HaCaT-Ras cells to various drugs**

HaCaT and HaCaT-Ras cells were treated with following drugs with specified concentration range and time duration. Results are stated in ‘Sensitivity’ column.

(O.C = Optimal Concentration at which all the cells died, o.m.p = over multiple passages, Note: Sensitivity of HaCaT cells to blasticidin and puromycin was not tested as they were already sensitive to G418 and Hygromycin.)

		HaCaT		HaCaT-Ras	
Drug	Concentration Range Tested (µg/mL)	Time Duration of Drug Exposure (Days)	Sensitivity	Time Duration of Drug Exposure (Days)	Sensitivity
Puromycin	1-10	Not Tested	Not Tested	5	Resistant
G418	100-1200	4	Sensitive O.C (1000 µg/mL)	17 o.m.p	Resistant
Hygromycin	100-1200	4	Sensitive O.C (600 µg/mL)	7 o.m.p	Resistant
Blasticidin	10-50	Not Tested	Not Tested	2 days	Sensitive O.C (20 µg/mL)

In summary, HaCaT-Ras cells were only sensitive to blasticidin, in contrast to HaCaT cells which were sensitive to both G418 and hygromycin.

### **Transfection, sorting of transfected cells, and selection of clones.**

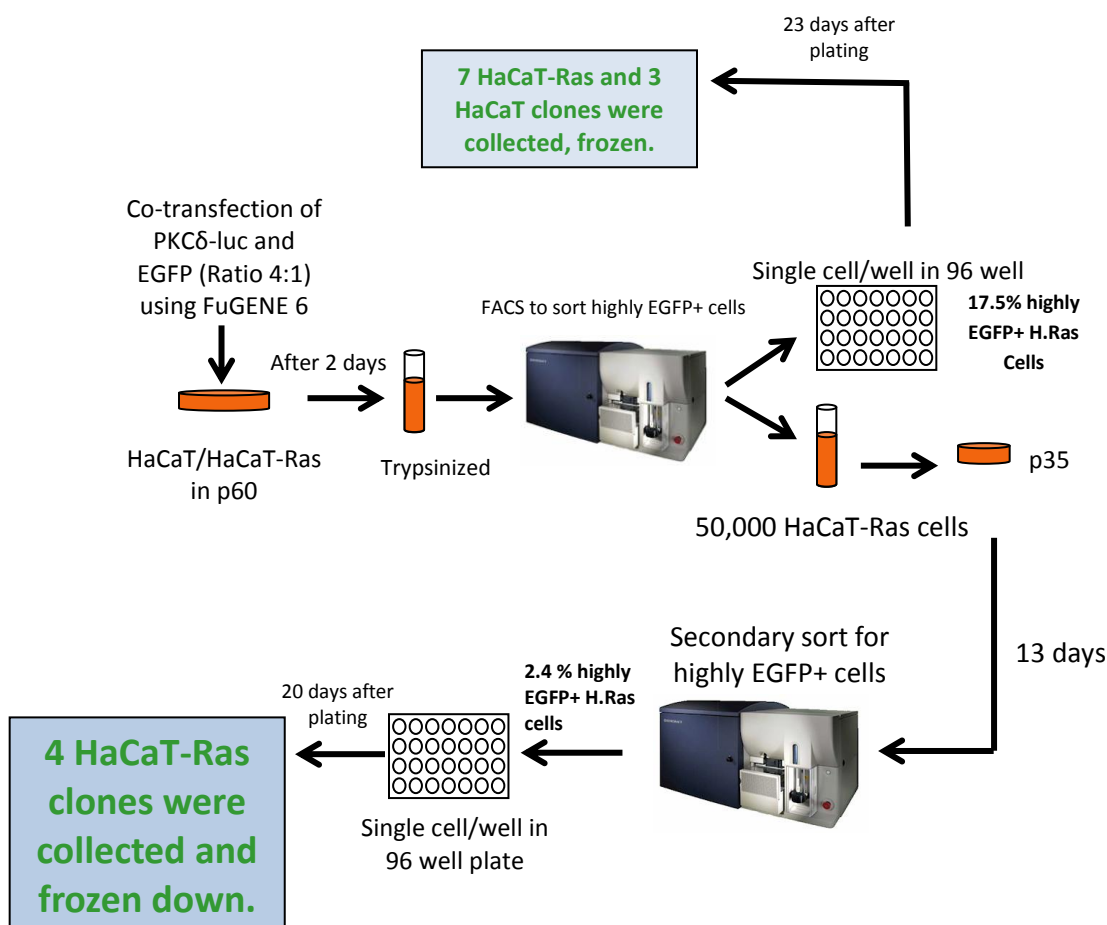
As an alternative approach to drug-selection, we decided to co-transfect HaCaT and HaCaT-Ras cells with PKC- $\delta$  promoter-reporter plasmid and an enhanced green fluorescent protein (EGFP) plasmid, and sort EGFP transfected cells by flow cytometry later on. Sorted EGFP<sup>+</sup> cells should express PKC- $\delta$  promoter-reporter as the quantity of PKC- $\delta$  reporter plasmid used for transfection was much higher than quantity of GFP plasmid. Also we proposed that EGFP fluorescence in cells could serve as an internal control for cell viability. Figure 6 illustrates the complete approach of cell sorting to develop stable, pGL3-hPKC $\delta$ -4.4 transfected cell lines.

We co-transfected pGL3-hPKC $\delta$ -4.4 plasmid in excess of pEGFP-C1 plasmid (4:1 ratio) in both HaCaT and HaCaT-Ras cells using FuGENE 6. 24 hours after transfection, we could visualize fluorescent HaCaT and HaCaT-Ras cells under fluorescent microscope, confirming that both the cells were transfected with pEGFP-C1. On the next day highly EGFP<sup>+</sup> HaCaT and HaCaT-Ras cells were sorted by FACS (Figure 7). 17.5 percent of HaCaT-Ras cells were highly EGFP<sup>+</sup> after primary bulk-sorting (Figure 7 B). The percentage of highly EGFP<sup>+</sup> HaCaT-Ras cells here reflects transfection efficiency of pEGFP-C1 in HaCaT-Ras cells. Sorted cells were plated in 96 well plates at single cell per well. Also 20,000 and 50,000 bulk sorted HaCaT and HaCaT-Ras cells respectively, were plated in p35 plates. 48 hours after plating, we could visualize a single fluorescent cell in many but not all wells of 96 well plates (Figure 8). After 11 days, we again sorted highly EGFP<sup>+</sup> cells from the HaCaT-Ras cells that were plated in p35 after first sorting.

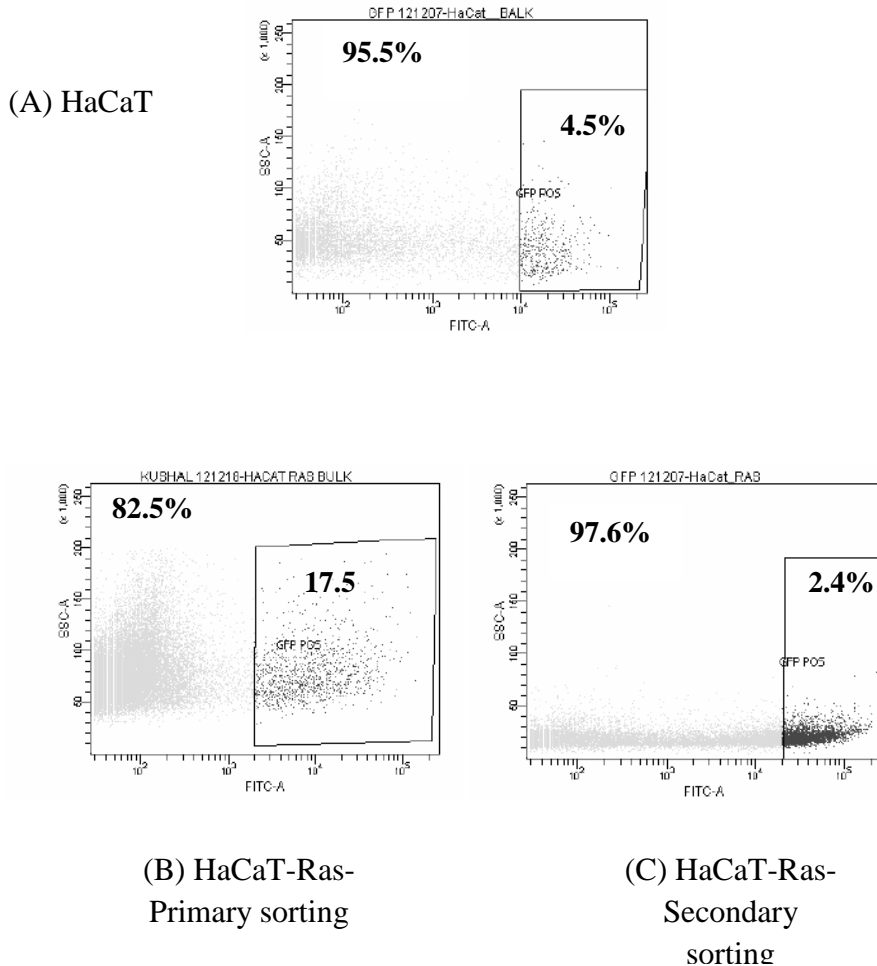
2.4 percent of HaCaT-Ras cells were highly EGFP<sup>+</sup> after secondary bulk-sorting (Figure 7 C). The percentage of highly EGFP<sup>+</sup> HaCaT-Ras cells here reflects percentage of HaCaT-Ras cells stably transfected with pEGFP-C1. Taking into account percentages of highly EGFP<sup>+</sup> cells from both the figures 7A and 7C (17.5 and 2.4 respectively), it could be interpreted that out of 100% highly EGFP<sup>+</sup> HaCaT-Ras cells sorted in primary sort, around 98% cells lost their EGFP expression by 10 days. Secondary sorted highly EGFP<sup>+</sup> HaCaT-Ras cells were also plated in 96 well plates at single cell per well to select clones.

We allowed sufficient time for clones to grow in 96 well plates, and selected clones which showed good growth rates. We selected 3 clones of HaCaT cells from sorted highly EGFP<sup>+</sup> HaCaT cells. 7 and 4 clones of HaCaT-Ras were selected from highly EGFP<sup>+</sup> HaCaT-Ras cells sorted for the first time and second time respectively. Selected clones were frozen down and stored in liquid nitrogen at -80° C.

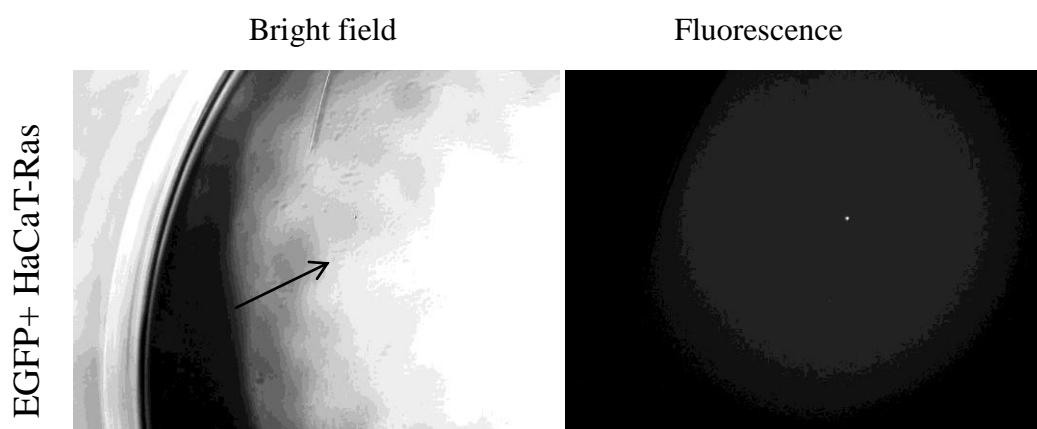




**Figure 6: Schematic of transfection and sorting of cells followed by selection of clones for screening:** pGL3-hPKCδ-4.4 and pEGFP-C1 were co-transfected into HaCaT and HaCaT-Ras cells in ratio of 4:1 using FuGENE 6. Ratio of 4:1 was used for amounts of pGL3-hPKCδ-4.4 and pEGFP-C1 so that probability of EGFP+ cells being transfected with pGL3-hPKCδ-4.4 is high. Flow cytometric cell sortings for highly EGFP+ cells were performed two times, followed by plating one highly EGFP+ cell per well of 96 well plates each time. After indicated time periods HaCaT-Ras and HaCaT clones were collected and frozen down at -80° C.



**Figure 7: Flow cytometric sorting of highly EGFP+ HaCaT and HaCaT-Ras cells:** In flow-cytometric analysis, percentages of highly pEGFP-C1+ cells were quantified in single cell population, gated from mixed cell population. 4.5% HaCaT cells (A) and 17.5% HaCaT-Ras cells (B) were found highly EGFP+ 48 hours post-transfection. This was followed by sorting, plating and expansion of highly EGFP+ cells in bulk. After 11 days, primary sorted HaCaT-Ras cells were sorted again and 2.4% HaCaT-Ras cells (C) were highly EGFP+.



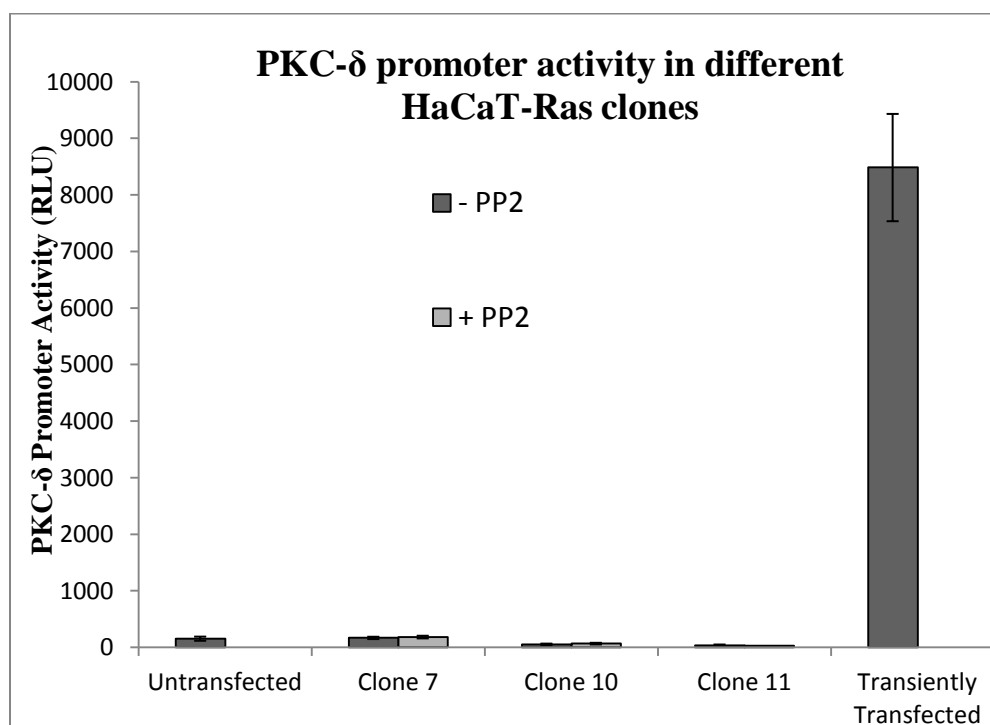
**Figure 8: Single EGFP+ HaCaT-Ras cell in a 96 well plate:** Image of a single EGFP+ HaCaT-Ras cell in 96 well plate observed 48 hours after plating. Objective: 4X

### Screening of selected clones

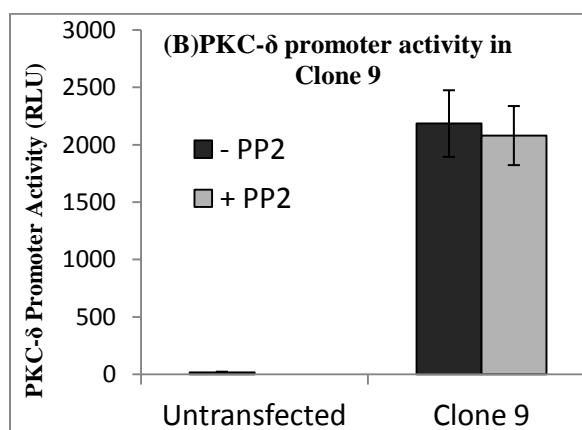
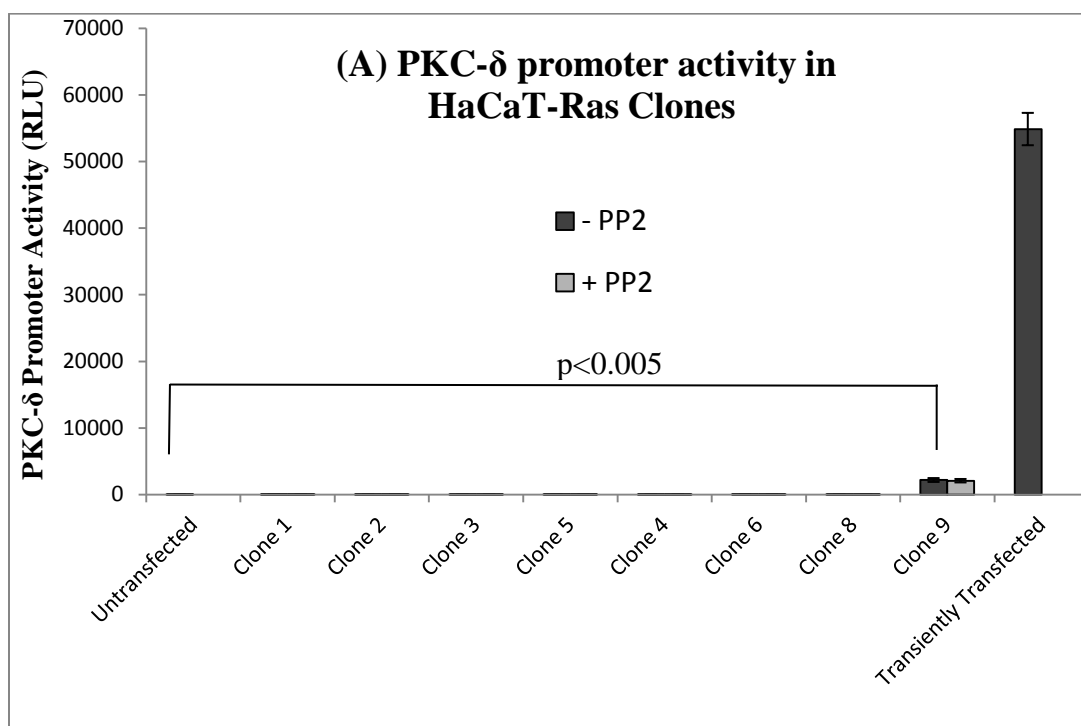
Approximately 60 days after freezing down the clones, all the frozen clones were thawed to screen for clones which were stably transfected. None of the HaCaT clones were EGFP+, however 4 out of 11 HaCaT-Ras clones were EGFP+ after thawing. The 4 EGFP+ HaCaT-Ras clones were clones 8, 9, 10 and 11.

Screening of HaCaT-Ras clones was performed in two batches (Figure 9 and 10), in which the HaCaT and HaCaT-Ras clones were plated in 96 well plates, and luciferase activity for each clone was measured in PHERAstar FS 72 hours after plating. Also, 24 hours after plating clones were treated with 250  $\mu$ M PP2 to see the inducibility of reporter activity in each clone. In screening, the only clone found stably transfected with pGL3-hPKC $\delta$ -4.4 was clone 9 (Figure 10 A). Difference between reporter activities of clone 9 and untransfected control was more than 100-fold and significant (Figure 10 B,

$p < 0.005$ ). On the other hand, reporter activity of clone 9 was drastically less compared to transiently transfected HaCaT-Ras cells, a positive control for this experiment (Figure 10 A). None of the HaCaT-Ras clones showed induction in PKC- $\delta$  reporter activity when treated with PP2 (Figure 9, 10). None of the HaCaT clones showed reporter activity higher than untransfected control in screening (Data not shown).



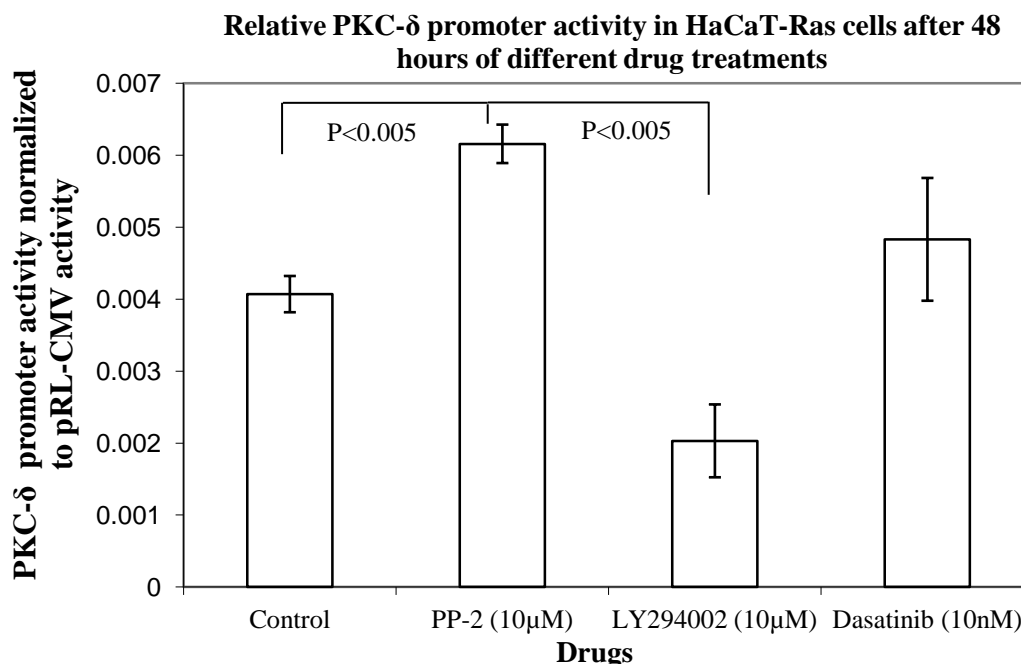
**Figure 9: HaCaT-Ras Clone Screening Batch 1:** HaCaT-Ras clones were plated in 96 well plates. 24 hours later clones were treated with 250  $\mu$ M PP2, and 48 hours following drug treatment, luminescence was measured in PHERAstar FS with untransfected HaCaT-Ras as negative control and transiently transfected HaCaT-Ras as positive control.  $n=9$  for untransfected, transiently transfected HaCaT-Ras cells and untreated clones,  $n=3$  for treated clones.  $n$ = number of replicates for any data point in one experiment. Error bars denote standard deviations.



**Figure 10: HaCaT-Ras Clone Screening Batch 2:** HaCaT-Ras clones were plated in 96 well plates. 24 hours later clones were treated with 250  $\mu$ M PP2, and 48 hours following drug treatment, luminescence was measured in PHERAstar FS. (A) Luciferase activity measured on all the clones with untransfected HaCaT-Ras as negative control and transiently transfected HaCaT-Ras as positive control (B) Re-plotting of luciferase data in (A) showing difference between luciferase activities of untransfected control and clone 9. Each data point is average of 6 replicates in one experiment. Error bars denote standard deviation.

### **PP2 induces PKC- $\delta$ promoter activity in HaCaT-Ras cells**

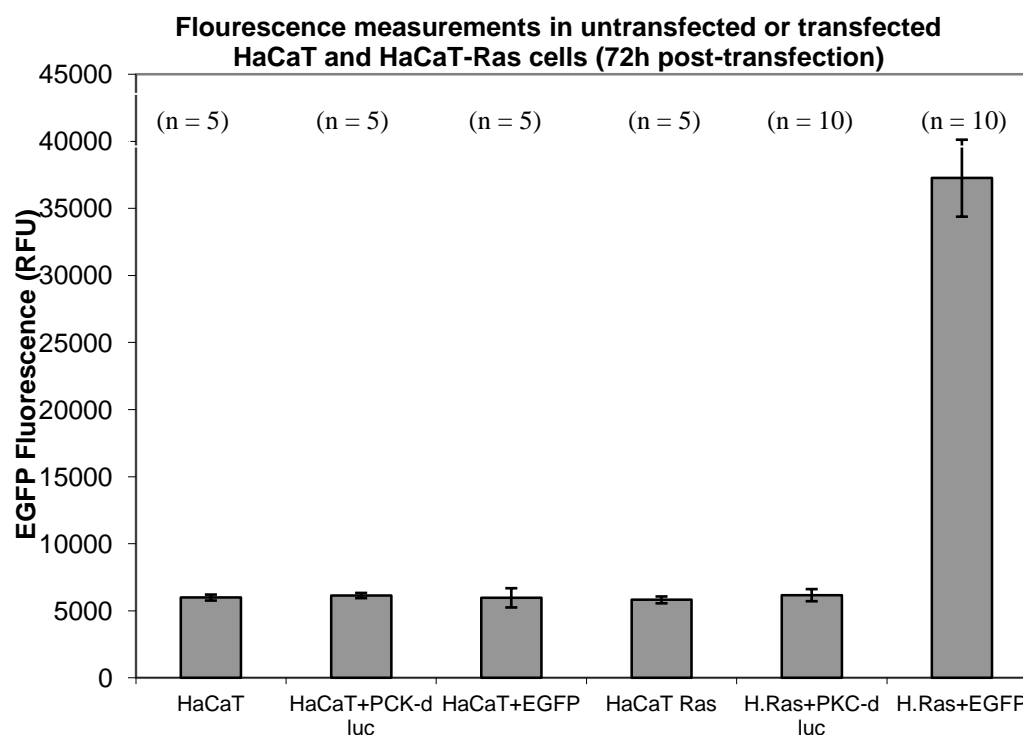
Next, we wanted to identify positive control compounds which could highly induce PKC- $\delta$  promoter activity in HaCaT-Ras cells to develop a robust high throughput assay having a high Z value. In order to do that, HaCaT-Ras cells were co-transfected with pGL3-hPKC $\delta$ -4.4 and pRL-CMV plasmids (in 16:1 ratio), and treated with PP2, LY294002 or Dasatinib one day post transfection. We hypothesized that src-family kinase inhibitors PP2 and Dasatinib, and PI3K inhibitor LY294002 would have potential to inhibit elements of Ras signaling pathway described earlier (Figure 4). 48 hours following drug treatments, firefly and renilla luciferase activities were measured by dual luciferase assay. As shown in Figure 11, PP2 (10  $\mu$ M) treatment showed 1.5-fold induction in PKC- $\delta$  promoter activity compared to untreated control. P value calculated for this difference of luciferase activities using Student's T test, was less than 0.005. As 0.05 is a pre-determined significance threshold, we considered this induction in reporter activity as statistically significant. LY294002 (10  $\mu$ M) treatment led to significant decrease in PKC- $\delta$  promoter activity, whereas Dasatinib (10 nM) treatment did not change it significantly (Figure 11).



**Figure 11: Dual luciferase assay on HaCaT-Ras cells after different drug treatments:** pGL3-hPKC $\delta$ -4.4 and pRL-CMV were co-transfected into HaCaT-Ras cells in relative amounts of 16:1 using TransIT 2020. 24 hours after transfection, cells were treated with indicated concentrations of PP2, LY294002, and Dasatinib. Dual luciferase assay was performed 48 hours following drug treatments. Each data point shown in figure is average of three replicates in one experiment. Error bars denote standard deviation.

### Optimization of PHERAstar FS for fluorescence and luminescence measurements

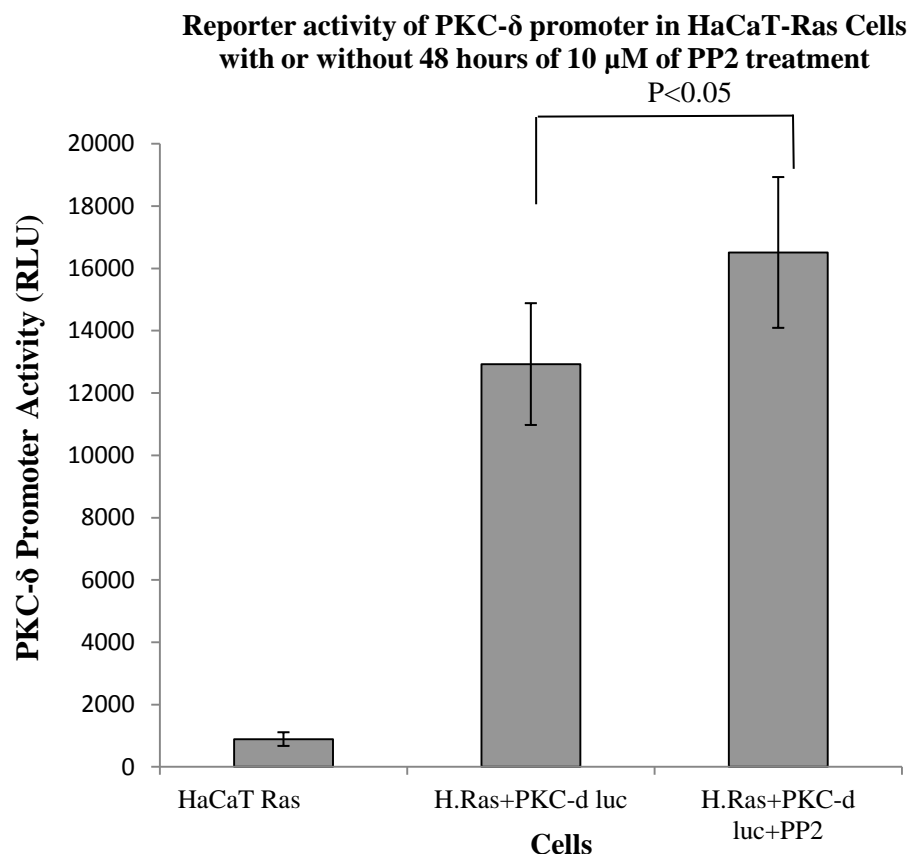
We sought to optimize PHERAstar FS for fluorescence and luminescence measurements in this project. We checked if PHERAstar FS had ability to detect fluorescent signals emitted by pEGFP-C1 transfected HaCaT-Ras cells in 96 well plates. As shown in Figure 12, EGFP transfected HaCaT-Ras cells showed very high fluorescence intensity compared to untransfected HaCaT-Ras cells in PHERAstar FS. In contrast, there was no significant difference observed between fluorescence of EGFP transfected and untransfected HaCaT cells, indicating very low transfection efficiency (Figure 12).



**Figure 12: Fluorescence measurement in PHERAstar FS:** HaCaT and HaCaT-Ras cells were transfected with indicated plasmids using FuGENE 6, and plated in a 96 well plate 24 hours after transfection. 48 hours after plating, fluorescence intensity was measured in PHERAstar FS. Note the high RFU value for EGFP transfected HaCaT-Ras cells. Here n = number of replicates for each data point in one experiment. Error bars denote standard deviations.

Figure 13 shows the luminescence measurements carried out on the PHERAstar FS on pGL3-hPKC $\delta$ -4.4 transfected HaCaT-Ras cells, with or without 10  $\mu$ M PP2 treatment. It is noticeable that luminescence measured in pGL3-hPKC $\delta$ -4.4 transfected HaCaT-Ras cells was more than 10-fold higher compared to untransfected control (Figure 13). Further, luciferase activity was significantly increased when transfected cells were treated with PP2 (Figure 13).

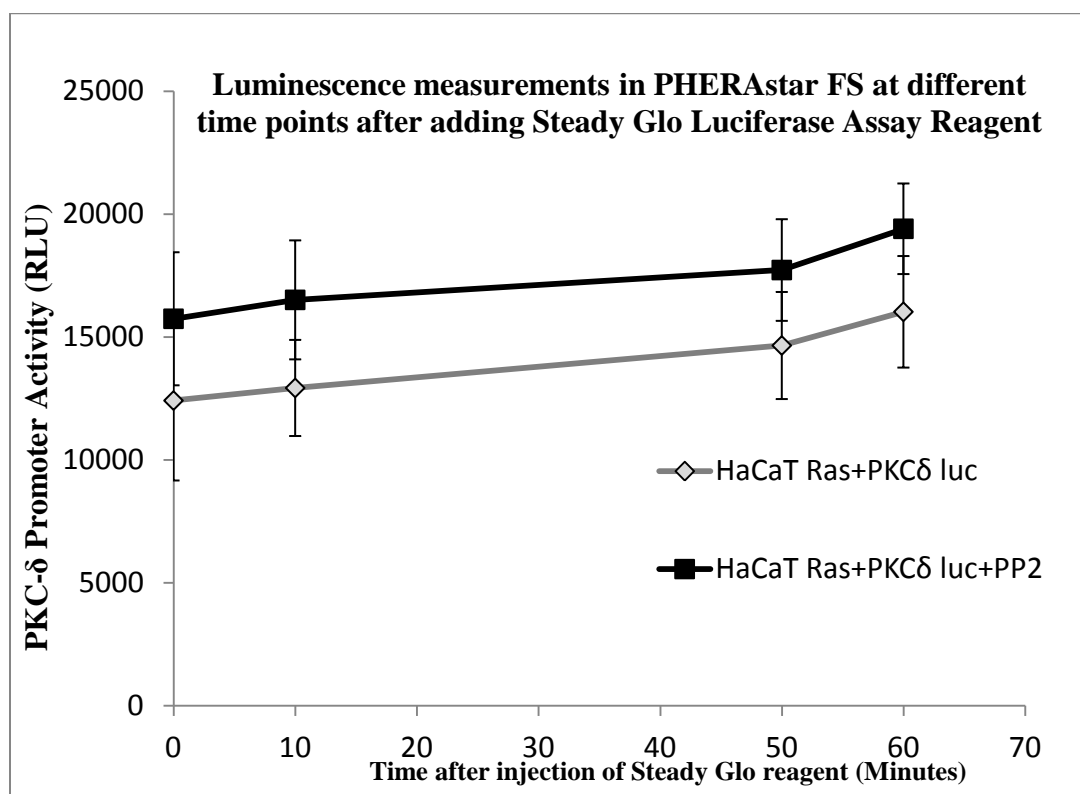




**Figure 13: Luminescence measurement in PHERAstar FS:** HaCaT and HaCaT-Ras cells were transfected with pGL3-hPKC $\delta$ -4.4 using FuGENE 6, and on next day, transfected cells were plated in a 96 well plate. 24 hours after plating, cells were treated with 10  $\mu$ M PP2. Luminescence was measured in PHERAstar FS 48 hours following drug treatment. Note RLU values in transfected cells with or without PP2 treatment.  $n = 5$  for all data points. Here  $n$  = number of replicates for each data point in one experiment. Error bars denote standard deviations.

To decide the optimal time point of reading luminescence in PHERAstar FS after addition of Steady-Glo Luciferase assay reagent, we measured luminescence at different time points after addition of assay reagent in addition to following manufacturer's recommended protocol of measuring luminescence 10 minutes after adding assay reagent (Figure 14). As seen in Figure 14, for both PP2 treated and untreated HaCaT-Ras cells

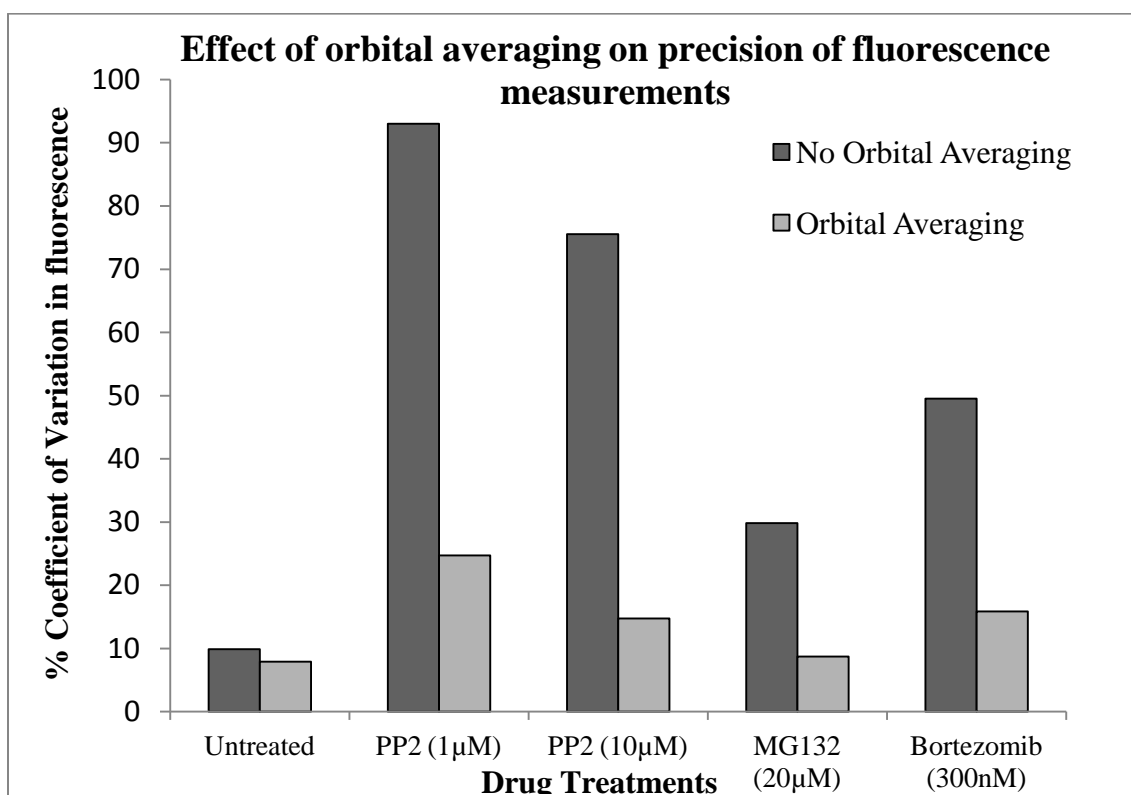
luciferase activity was fairly similar over time. Although it increased to small extent after 50 minutes, increases in PP2 treated and untreated cells were similar (Figure 14). As measuring luminescence after longer waiting times (50, 60 minutes) did not offer any advantages, we chose 10 minutes as an optimal time point for reading luminescence after adding reagent.



**Figure 14: Luminescence measurement in PHERAstar FS over different time points:** HaCaT-Ras cells were transfected with pGL3-PKC- $\delta$ -4.4 plasmid using FuGENE 6, and on next day transfected cells were plated in a 96 well plate. 24 hours after plating, cells were treated with 10  $\mu$ M PP2. 48 hours following drug treatment, luminescence was measured in PHERAstar FS 0, 10, 50 and 60 minutes after adding Steady-Glo Luciferase assay reagent. RLU values shown in the graph are averages of five replicates in one experiment.

We also carried out experiment to determine best optic settings in PHERAstar FS to measure signals from a 96 well plate, and obtained the following results for three important optic parameters of PHERAstar FS.

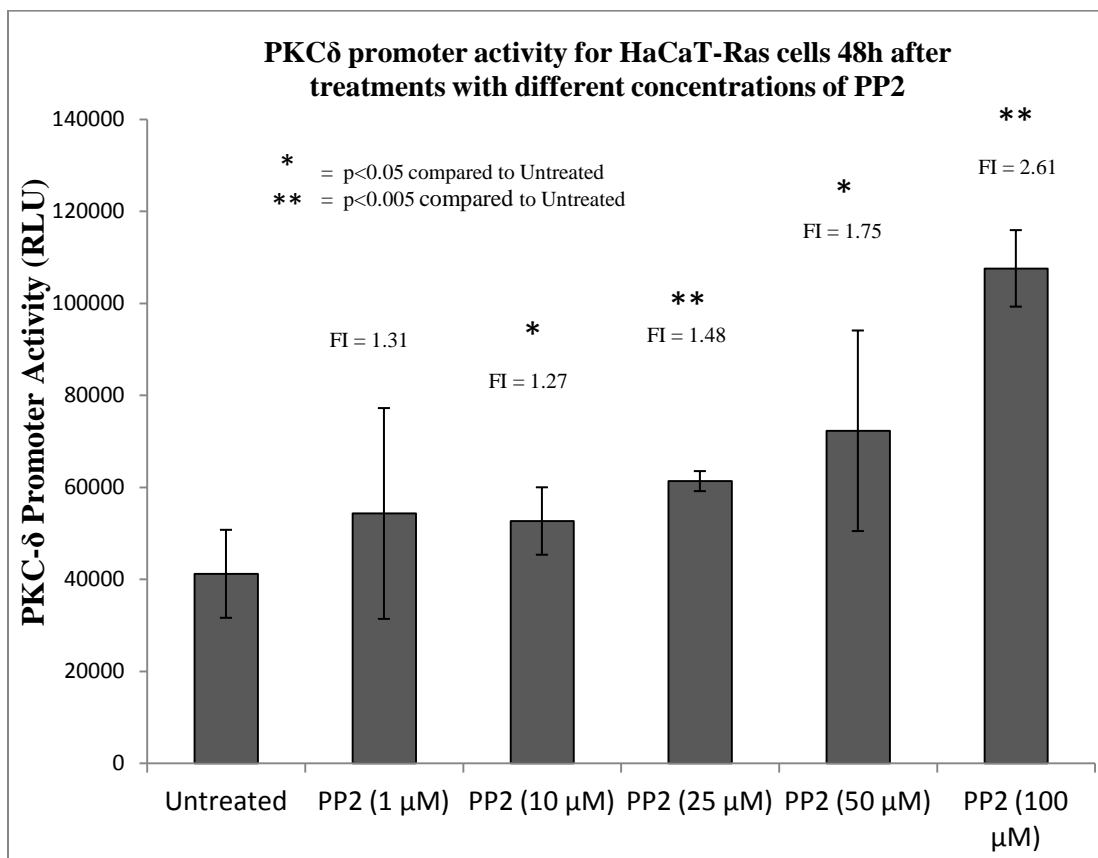
1. Measurements by 'Bottom Optics' were more sensitive and accurate compared to those by 'Top Optics' (Data not shown).
2. Using 'Orbital Averaging' during read-outs increased agreeability of readings amongst replicates of similar groups (Figure 15). However, orbital averaging did not affect magnitude of signals.
3. We read fluorescence signals on PHERAstar FS using different numbers of flashes per well in the range of 0 to 159, and found that increasing number of flashes per well increased accuracy of read-outs (Data not shown). So we used 159 flashes per well to measure fluorescence in PHERAstar FS.



**Figure 15: Orbital averaging in PHERAstar FS improves precision of fluorescence measurements:** HaCaT-Ras cells were transfected with pGL3-PKC- $\delta$ -4.4 plasmid using FuGENE 6, and on next day transfected cells were plated in a 96 well plate. 24 hours after plating, cells were treated with indicated drugs. 48 hours following drug treatment, luminescence was measured in PHERAstar FS. %CV was calculated from fluorescence intensities of three replicates in one experiment.

**Higher doses of PP2 lead to higher induction in PKC- $\delta$  reporter activity**

To characterize dose-response effect of PP2 on HaCaT-Ras cells, we treated pGL3-PKC- $\delta$ -4.4 transfected HaCaT-Ras cells with increasing doses of PP2 and found that increase in dose of PP2 results in higher induction in PKC- $\delta$  promoter activity (Figure 16). Highest fold induction in reporter activity compared to control was 2.61-fold for 100  $\mu$ M PP2 (Figure 16). Moreover, there was no peak effect observed in the dose-response curve (Figure 16, note last two columns).

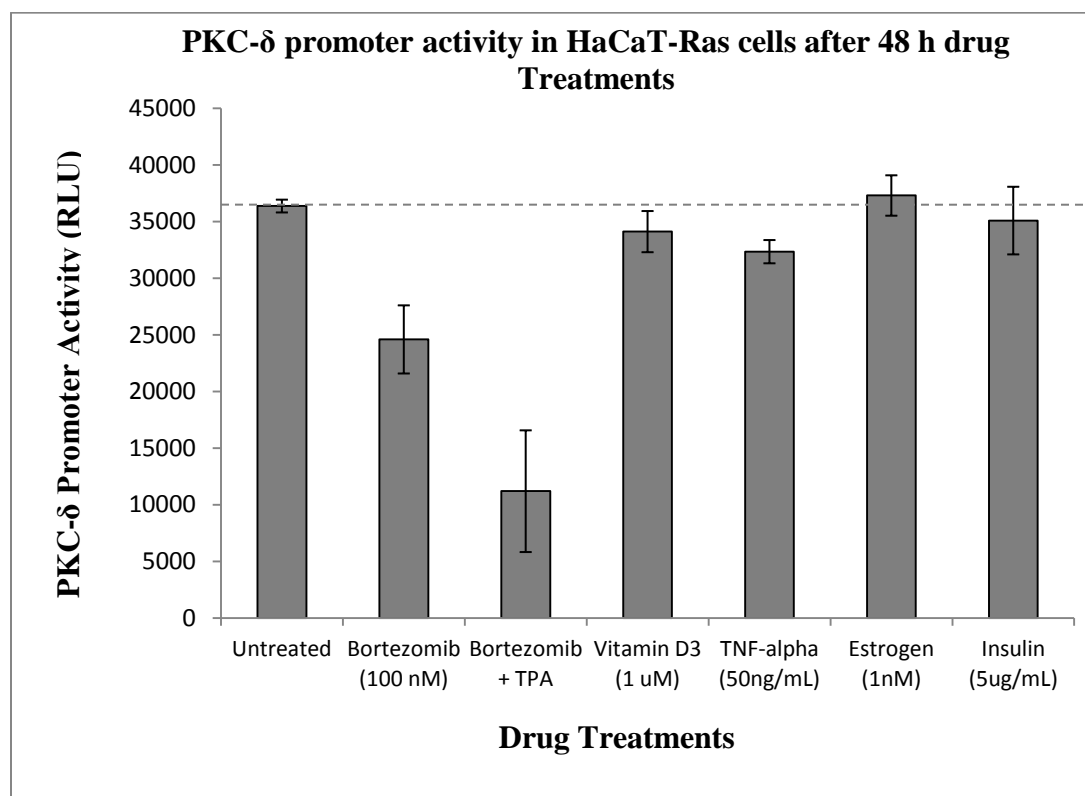


**Figure 16: PP2 Dose-Response Relationship:** HaCaT-Ras cells were transfected with pGL3-PKC- $\delta$ -4.4 using FuGENE 6, and plated in 96 well plate on the next day. 24 hours after plating, cells were treated with different concentrations of PP2. 48 hours following drug treatment, luciferase activity was measured in PHERAstar FS. n= 4 for PP2 (1  $\mu$ M), n=6 for other data points, where n= number of replicates for data point in one experiment. FI = Fold Induction. Error bars denote standard deviations.

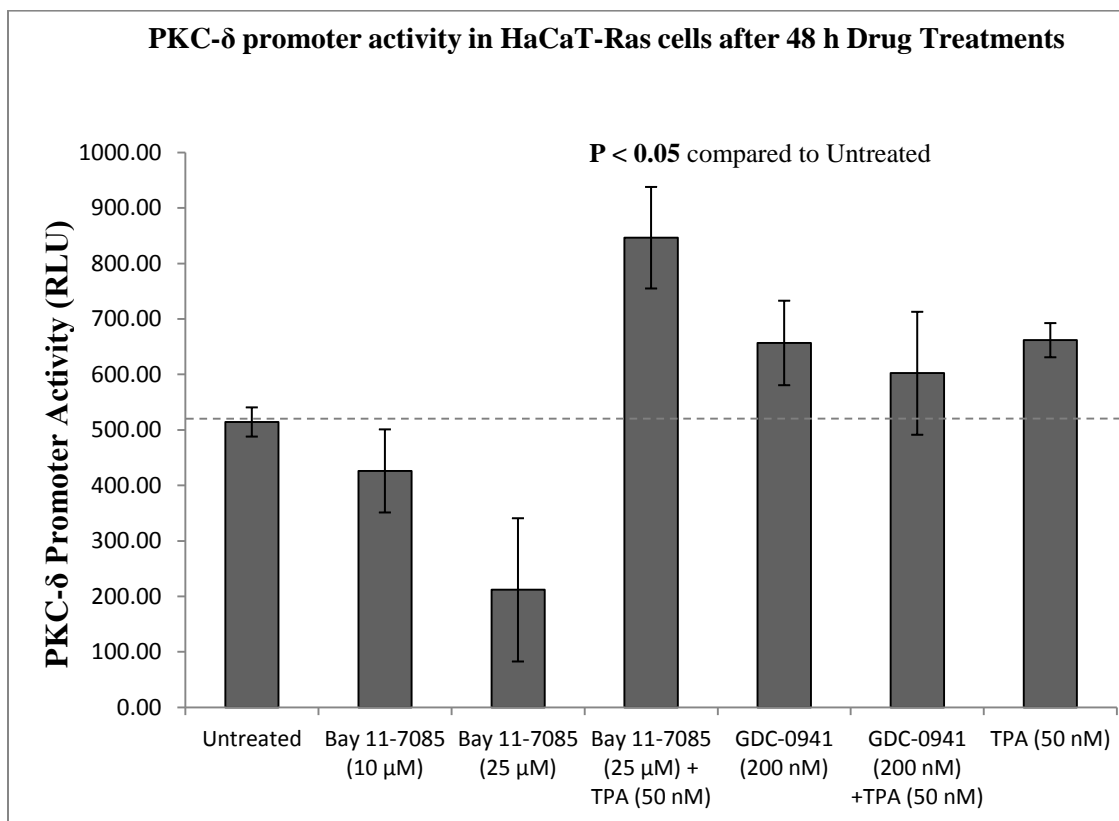
### Combination of Bay 11-7085 and TPA induces PKC- $\delta$ reporter activity

In experiments similar to the one described earlier (Figure 16), we tested different compounds and combination of compounds for their ability to induce PKC- $\delta$  reporter activity (Figure 17, 18). The compounds tested included proteasome inhibitor Bortezomib (61), NF- $\kappa$ B inhibitor Bay 11-7085, PI3K inhibitor GDC-0941 which were used to inhibit elements of proposed Ras signaling pathway earlier (Figure 4) and TNF- $\alpha$ ,

Vitamin D3, Estrogen, Insulin and TPA which were shown to induce PKC- $\delta$  gene expression in other studies (24, 62-65). As evident in Figure 18, combination of 25  $\mu$ M Bay 11-7085 and 50 nM TPA gave the highest induction in reporter activity amongst all compounds or combination of compounds tested.



**Figure 17: Testing different compounds for their ability to induce PKC- $\delta$  promoter activity 1:** HaCaT-Ras cells were transfected with pGL3-PKC- $\delta$ -4.4 using FuGENE 6, and plated in 96 well plate on the next day. 24 hours after plating, cells were treated with indicated concentrations of Bortezomib, Vitamin D3, TNF- $\alpha$ , Estrogen, and Insulin. 48 hours following drug treatment, luciferase activity was measured in PHERAstar FS. n= 9 for untreated, n=3 for other data points, where n= number of replicates for data point in one experiment. Dotted line indicates basal PKC- $\delta$  reporter activity in untreated HaCaT-Ras cells on graph. Error bars denote standard deviations.



**Figure 18: Testing different compounds for their ability to induce PKC- $\delta$  promoter activity 2:** HaCaT-Ras cells were transfected with pGL3-PKC- $\delta$ -4.4 using FuGENE 6, and plated in 96 well plate on the next day. 24 hours after plating, cells were treated with indicated concentrations of Bay 11-7085, Bay 11-7085+TPA, GDC-0941, GDC-0941+TPA and TPA. 48 hours following drug treatment, luciferase activity was measured in PHERAstar FS.  $n=9$  for untreated,  $n=3$  for other data points, where  $n$ = number of replicates for data point in one experiment. Dotted line indicates basal PKC- $\delta$  reporter activity in untreated HaCaT-Ras cells on graph. Error bars denote standard deviations.

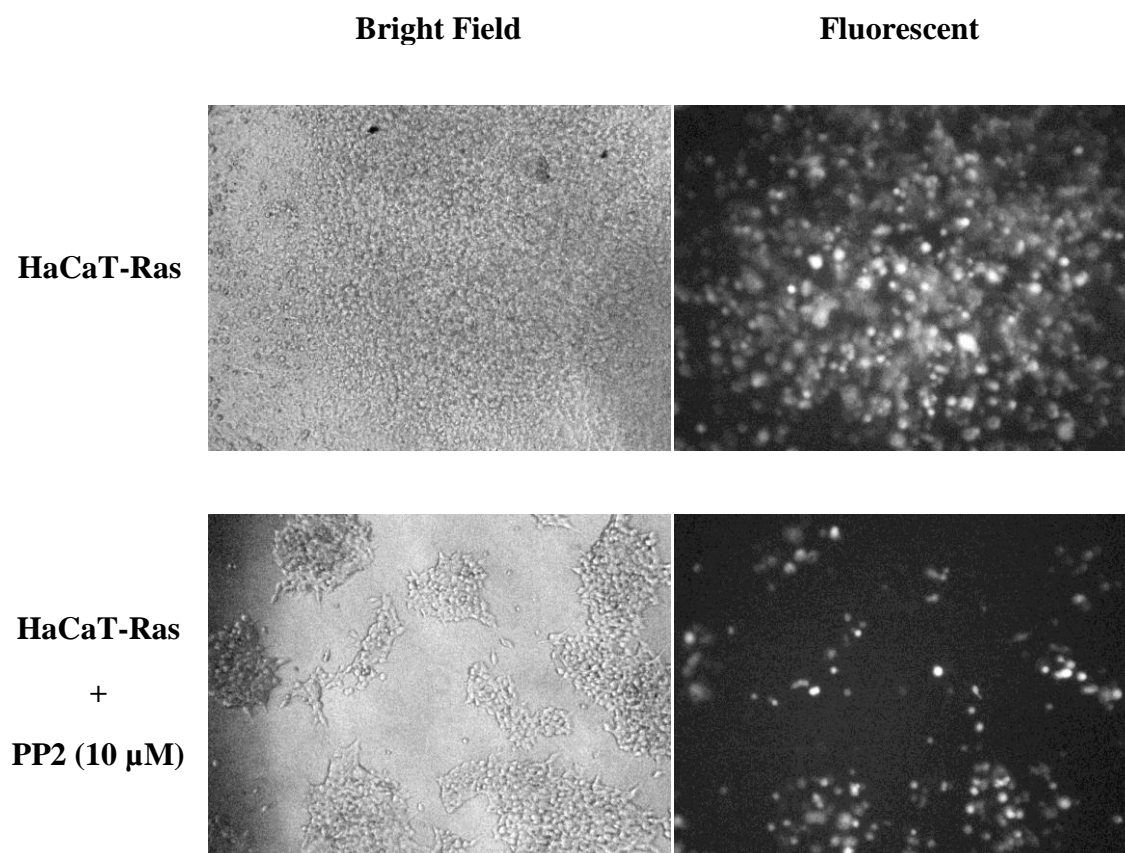


### **Z value calculations**

Z values for the two candidate assays in which positive control compounds showed highest induction in PKC- $\delta$  reporter activity, were calculated by equation described earlier (59). The first assay, consisting of combination of Bay 11-7085 and TPA as a positive control (Figure 18, column 1 as a negative control, column 4 positive control), gave Z value of -0.06 after analysis. Following interpretations for Z values described in section 1.7 in background, this assay was unacceptable for high throughput screening. Second assay, which employed PP2 as a positive control (Figure 16, column 1 as a negative control, column 6 positive control), gave Z value of 0.19. Z value of 0.19 could be interpreted as a doable assay (Table 1). However, none of the candidate assays had our desired Z value of 0.5 or above to generate an excellent assay. So in order to obtain higher Z values, we attempted to find ways to increase the induction in reporter activity with drug treatments and decrease the variability of luminescence.

### **Treatment of HaCaT-Ras cells with PP2 reduces number of attached cells**

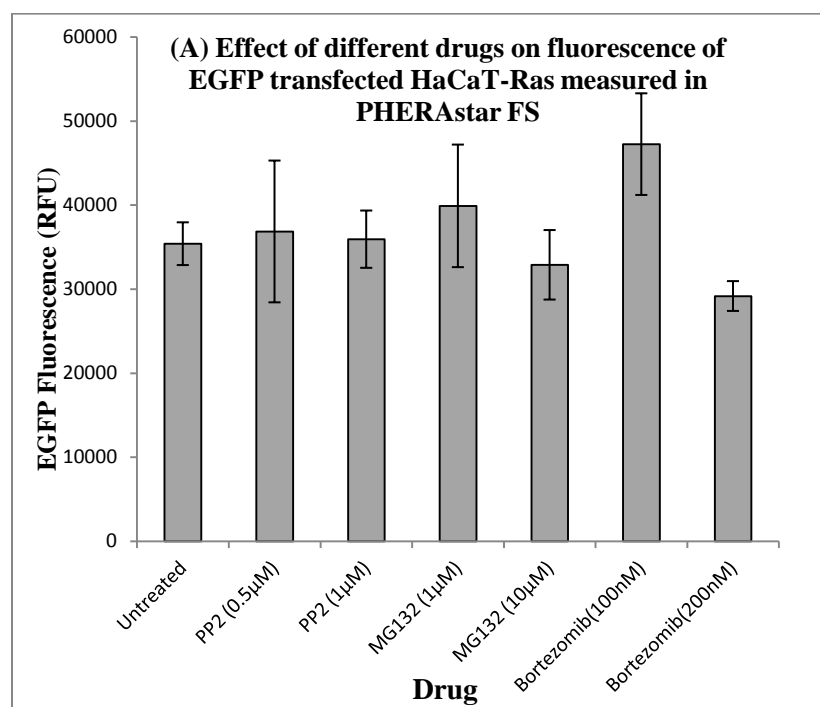
If drug treatments had any effect on number or viability of HaCaT-Ras cells, those effects would also have effects on the luciferase activities of treated cells. Therefore it was important to know whether or not drugs being used in this project had any effects on number or viability of HaCaT-Ras cells, and if they did, to find out a convenient way to normalize the luminescence data with cell number or viability. When EGFP transfected HaCaT-Ras cells were treated with 10  $\mu$ M PP2 for 48 hours, they showed formation of clusters and reduction in number of attached cells (Figure 19).



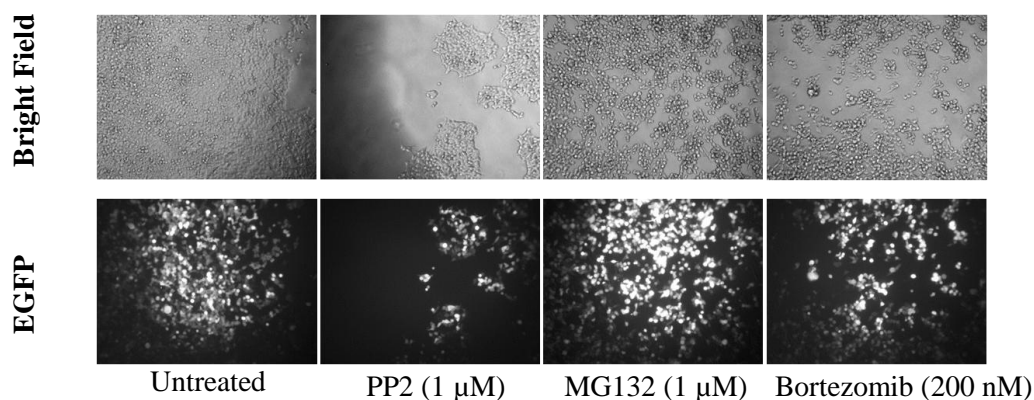
**Figure 19: Effect of PP2 on morphology and number of attached HaCaT-Ras cells:** HaCaT-Ras cells were transfected with EGFP using FuGENE 6, and plated in 96 well plate on the next day. 24 hours after plating, cells were treated with 10  $\mu$ M PP2. Images shown here were captured in fluorescent microscope 48 hours after PP2 treatment. Objective: 4X

**EGFP fluorescence does not reflect number of attached HaCaT-Ras cells observed  
on plate**

When we treated EGFP transfected HaCaT-Ras cells with drugs PP2, Bortezomib and MG132 (another proteasome inhibitor), we found poor correlation between number of attached HaCaT-Ras cells observed under microscope and fluorescence measured in PHERAstar FS after drug treatments (Figure 20). Specifically, with 1  $\mu$ M PP2 treatment, reduction in number of attached cells observed under microscope (Figure 20 B, second column) was not reflected in fluorescence measurement as a reduction in fluorescence intensity (Figure 20 A, third bar). This was also true for 1  $\mu$ M MG132 treatment (Figure 20 A-fourth lane, B -third image). Thus EGFP fluorescence was not a reliable method to normalize for cell number and viability.



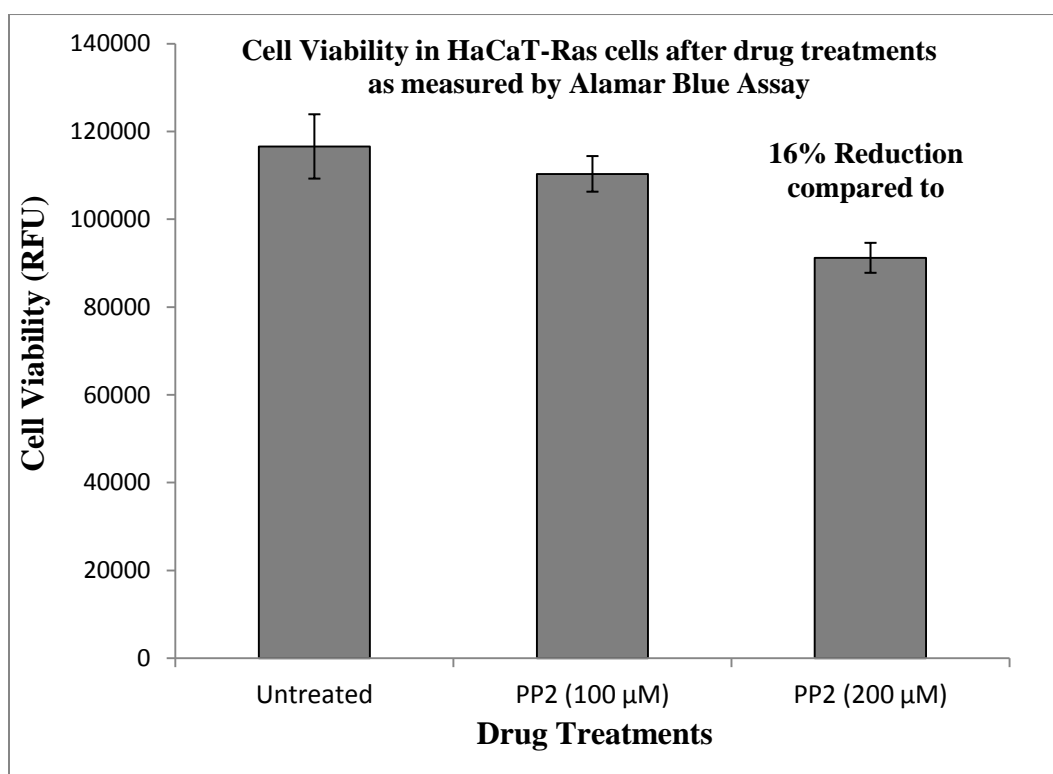
**(B) Effect of different drugs on EGFP+ HaCaT-Ras cells after 24 hours**



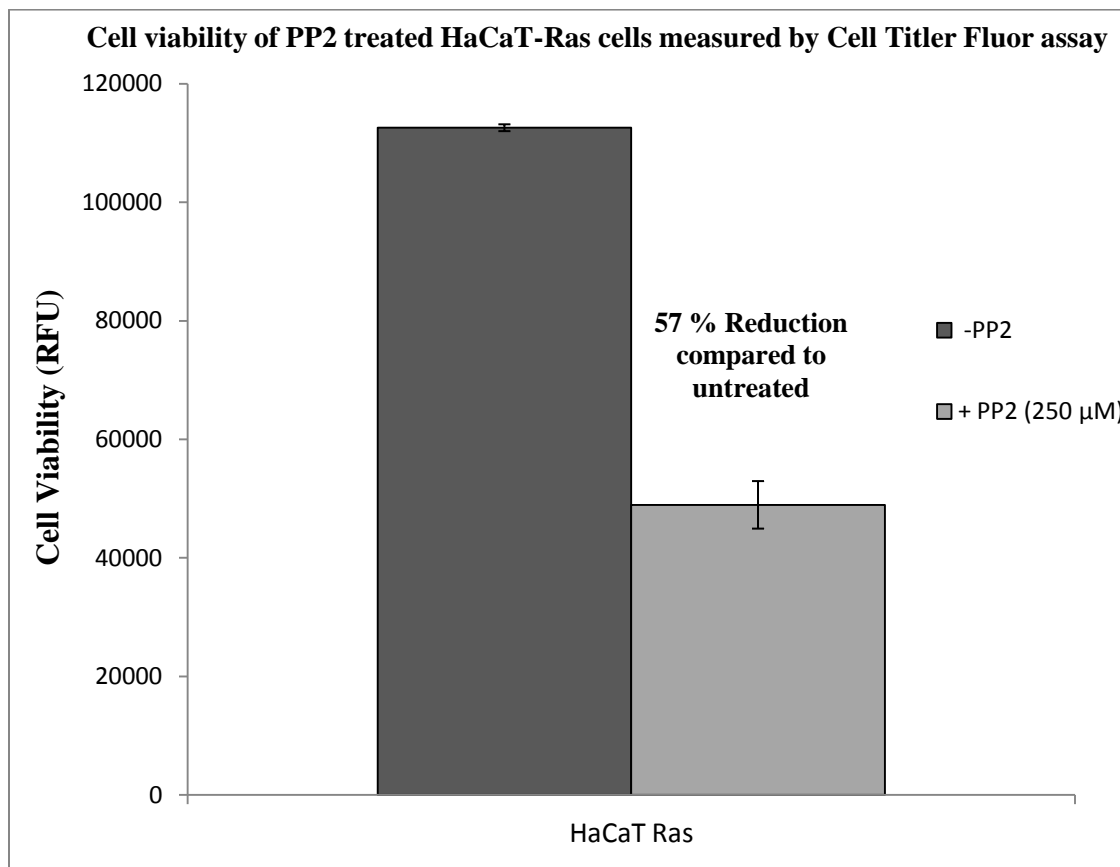
**Figure 20: Poor correlation between fluorescence read by PHERAstar FS and number of attached cells observed for HaCaT-Ras cells treated with different drugs:** Treatments with the indicated drugs were started on HaCaT-Ras cells, co-transfected with pGL3-hPKC- $\delta$ -4.4 kb and EGFP 24 hours post-transfection. Then (A) Fluorescence was measured using PHERAstar FS, 48 hours post drug treatments. Error bars denote standard deviations (B) Cells were observed under fluorescent microscope, 24 hours after initiation of drug treatments. Shown are the images of PP2 (1 μM), MG132 (1 μM), and Bortezomib (200 nM) treated cells. Objective: 4X. n=6 for Untreated, n=3 for all drug treatments, n= number of replicates for any data point in one experiment.

### Viability of HaCaT-Ras cells decreases after PP2 treatment

We used different methods of measuring cell viability to see if reduction in number of attached cells observed after PP2 treatment (Figure 20 B) is actually reduction in cell viability or not. Indeed, PP2 treatment decreased number of viable HaCaT-Ras cells as measured by Alamar Blue and Cell-Titer Fluor Cell Viability Assays (Figures 21,22). Reduction in cell viability assay was 16% using Alamar Blue after 200  $\mu$ M PP2 treatment (Figure 21), whereas it was 57% using Cell-Titler Fluor Cell Viability assay after 250  $\mu$ M PP2 treatment (Figure 22).



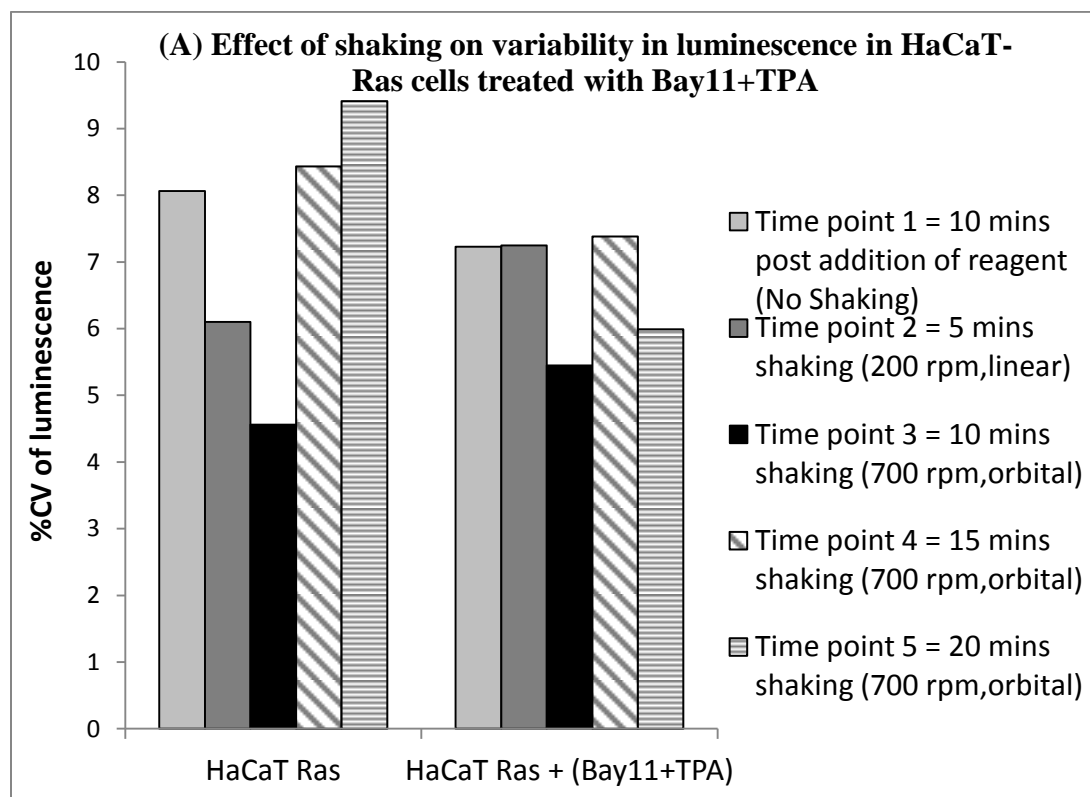
**Figure 21: Cell viability measurements using Alamar Blue Assay:** pGL3-hPKC- $\delta$ -4.4 kb transfected HaCaT-Ras cells were plated in 96 well plate. 24 hours after plating, cells were treated with PP2. 48 hours after drug treatments, cell viability was measured using Alamar Blue assay following manufacturer's protocol. n=6 for all data points, where n = number of replicates for any data point in one experiment. Error bars denote standard deviations.



**Figure 22: Cell viability measurements using Cell-Titler Fluor Viability Assay:** HaCaT-Ras cells were plated in 96 well plate. 24 hours after plating, cells were treated with PP2. 48 hours after drug treatments, cell viability was measured using Cell-Titler Fluor Viability assay following manufacturer's protocol.  $n=2$  for all data points, where  $n$  = number of replicates for any data point in one experiment. Error bars denote range.

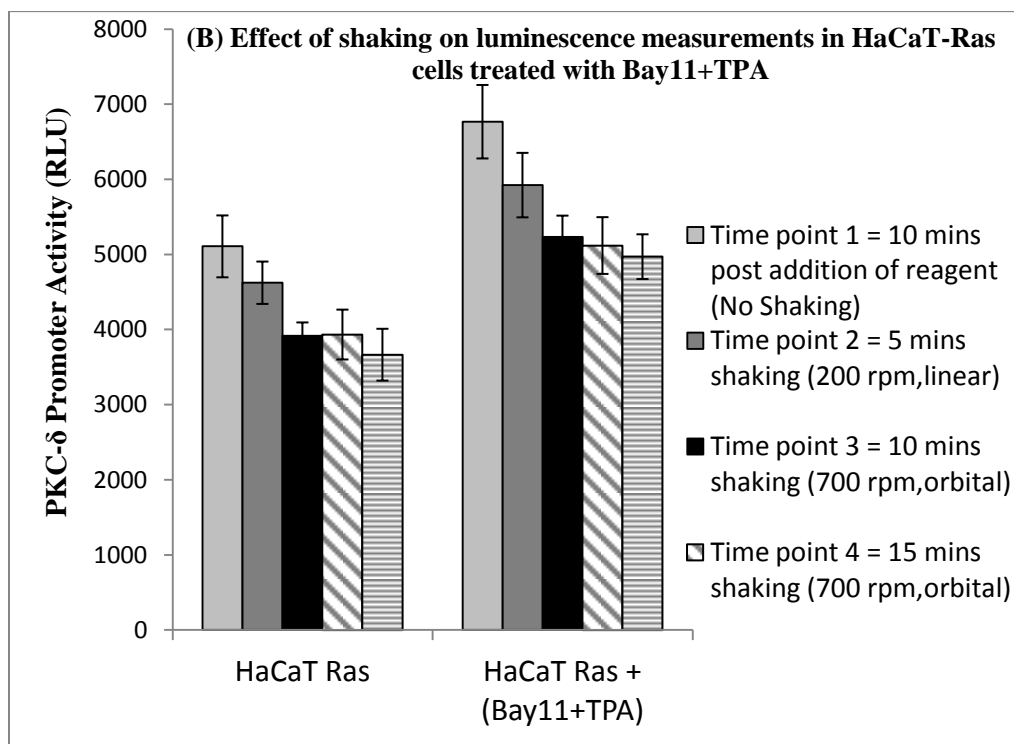
### **Effect of shaking on variability of luminescence**

To reduce variability in luminescence read-outs in PHERAstar FS, we employed a shaking step in our protocol and assessed how it affects %CV (Co-efficient of Variation) of luminescence read-outs. We saw reduction in %CV after shaking the plate for up to 10 minutes after measuring luminescence by Steady-Glo Luciferase Assay protocol (reading luminescence 10 minutes after addition of assay reagent without shaking the plate) (Figure 23 A). Shaking for more than 10 minutes after first measurement resulted in increase in %CV (Figure 23 A, time point 4 and 5). Figure 23 B depicts the luminescence values read on PHERAstar FS in this experiment.



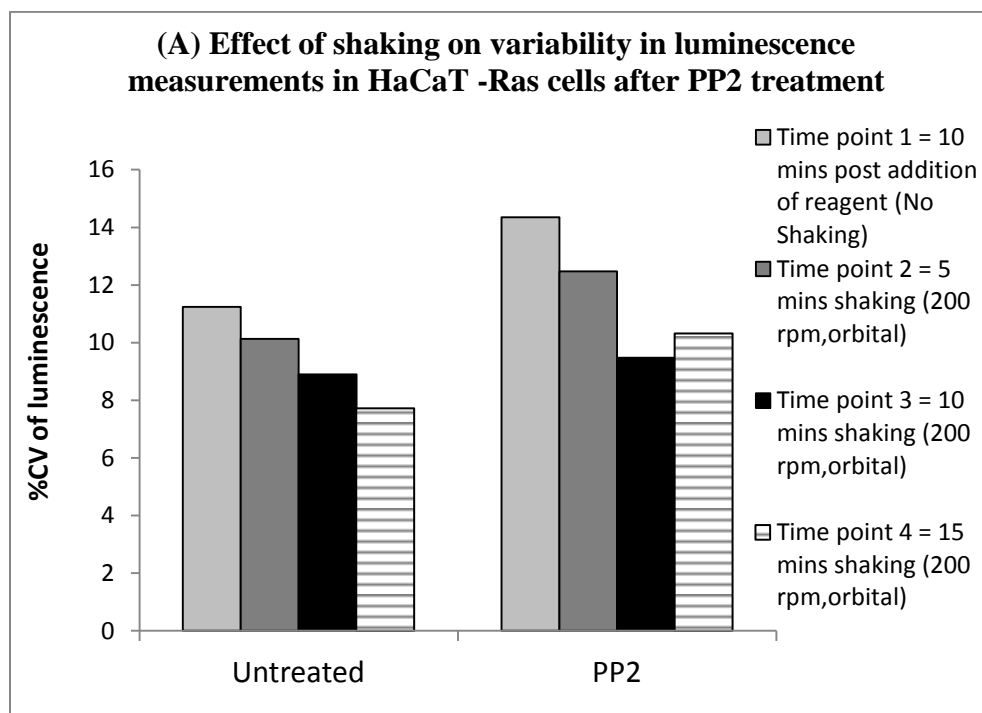
**Figure 23 (A): Shaking reduces variability of luminescence read-outs in PHERAstar FS:** pGL3-hPKC- $\delta$ -4.4 kb transfected HaCaT-Ras cells were plated in 96 well plate. 24 hours after plating, cells were treated with combination of 25  $\mu$ M Bay 11-7085 and 50 nM TPA. 48 hours after drug treatments, luminescence was measured in PHERAstar FS at different time points described in figures. Briefly, for first time point, company protocol was followed to measure luciferase activity without shaking and each of subsequent measurements included 5 minutes shaking period. (A) shows %CV calculated from (B) luminescence values for all groups (See next page). For each time point cumulative shaking time is mentioned in figure. Speed and motion of shaking of most recent shaking is mentioned in brackets.



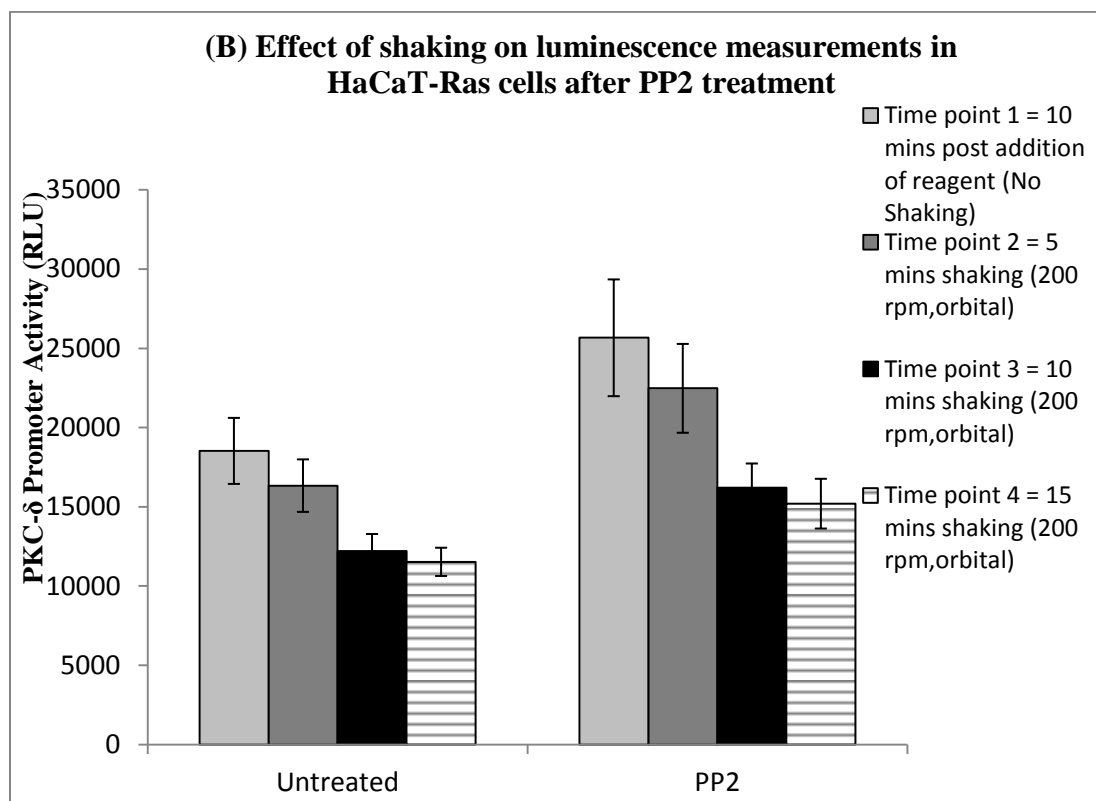


**Figure 23 (B): Shaking reduces variability of luminescence read-outs in PHERAstar FS:** pGL3-hPKC- $\delta$ -4.4 kb transfected HaCaT-Ras cells were plated in 96 well plate. 24 hours after plating, cells were treated with combination of 25  $\mu$ M Bay 11-7085 and 50 nM TPA. 48 hours after drug treatments, luminescence was measured in PHERAstar FS at different time points described in figures. Briefly, for first time point, company protocol was followed to measure luciferase activity without shaking and each of subsequent measurements included 5 minutes shaking period. (B) shows luminescence values for all groups.  $n = 6$  for all data points, where  $n$  = number of replicates for any data point in one experiment. For each time point cumulative shaking time in mentioned in figure. Speed and motion of shaking of most recent shaking is mentioned in brackets. Error bars denote standard deviations.

We also looked at the effect of shaking on variability of luminescence in HaCaT-Ras cells treated with PP2. We found that increase in shaking time results in decrease in %CV of luminescence (Figure 24 A). Again, we noticed that at time point 3 (after 10 minutes of shaking) %CV was low for both untreated and PP2 treated HaCaT-Ras cells, but increasing shaking time beyond that resulted in increase in %CV for PP2 treated cells (Figure 24 A).

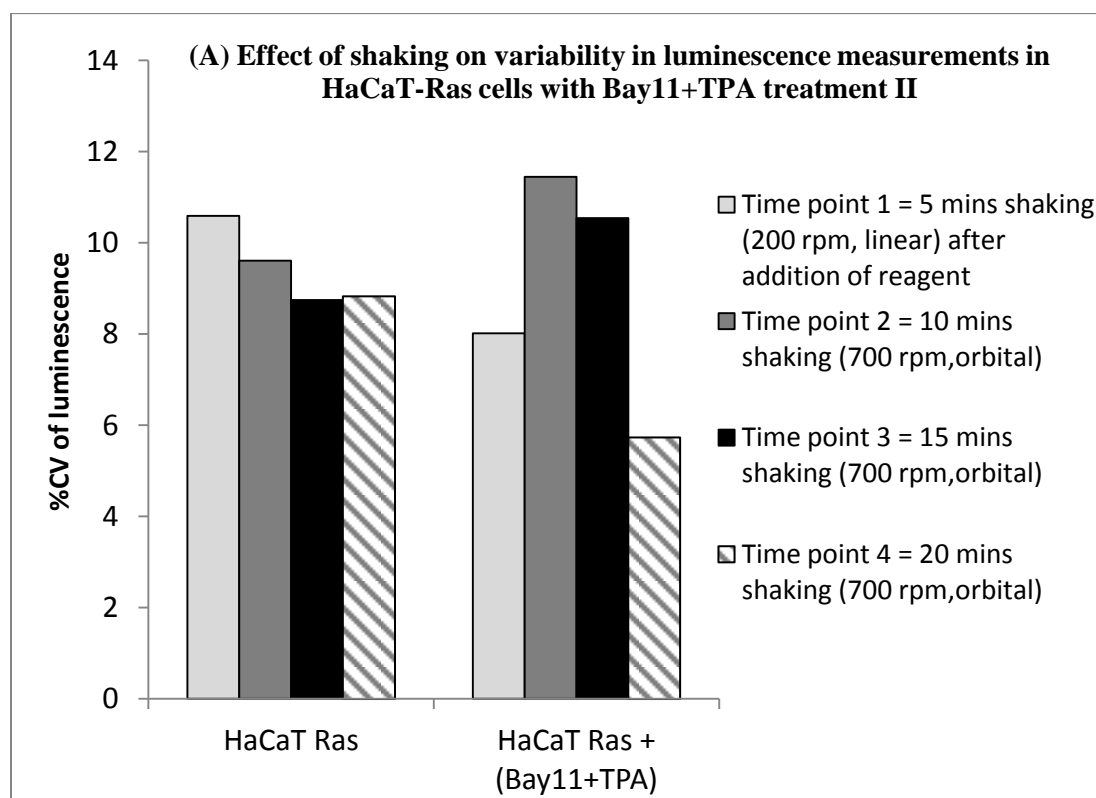


**Figure 24 (A): Effect of shaking on PP2 treated HaCaT-Ras cells:** pGL3-hPKC- $\delta$ -4.4 kb transfected HaCaT-Ras cells were plated in 96 well plate. 24 hours after plating, cells were treated with 250  $\mu$ M PP2. 48 hours after drug treatments, luminescence was measured in PHERAstar FS at different time points indicated in figures. Briefly, for first time point, company protocol was followed to measure luciferase activity without shaking and each of subsequent measurements included 5 minutes shaking period. (A) shows %CV calculated from (B) luminescence values for all groups (See next page). For each time point cumulative shaking time in mentioned in figure. Speed and motion of shaking of most recent shaking is mentioned in brackets.

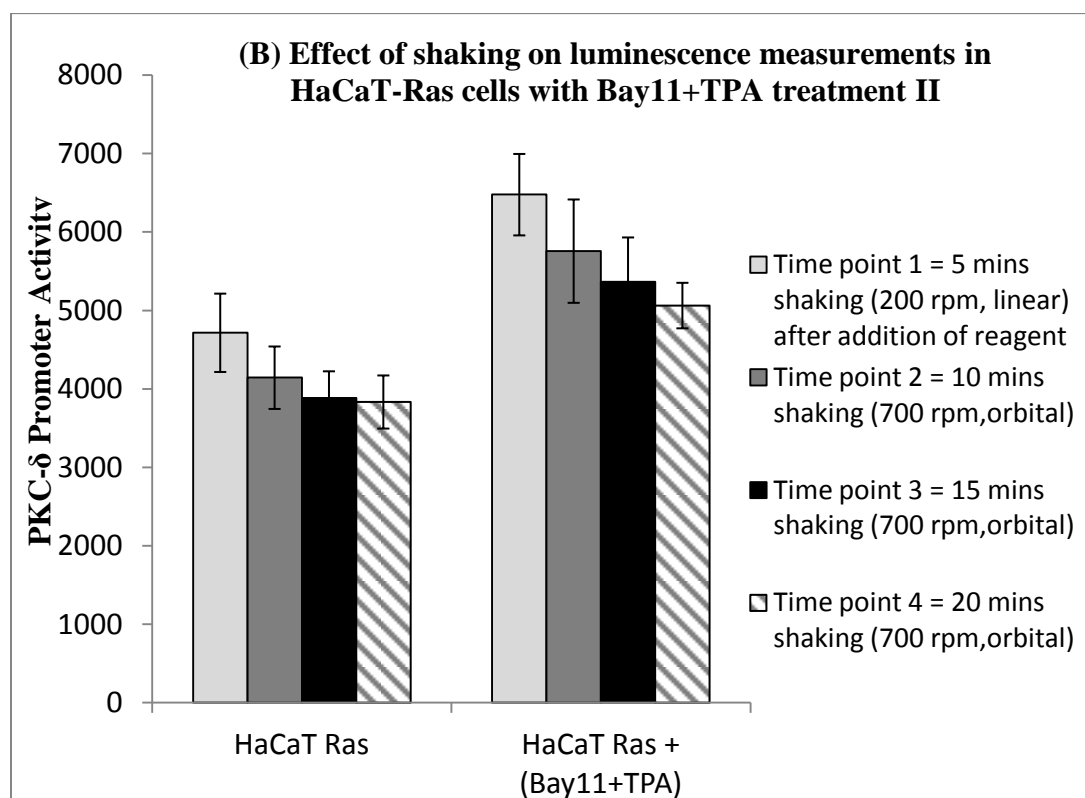


**Figure 24 (B): Effect of shaking on PP2 treated HaCaT-Ras cells:** pGL3-hPKC- $\delta$ -4.4 kb transfected HaCaT-Ras cells were plated in 96 well plate. 24 hours after plating, cells were treated with 250  $\mu$ M PP2. 48 hours after drug treatments, luminescence was measured in PHERAstar FS at different time points indicated in figures. Briefly, for first time point, company protocol was followed to measure luciferase activity without shaking and each of subsequent measurements included 5 minutes shaking period. (B) shows luminescence values for all groups.  $n = 6$  for all data points, where  $n$  = number of replicates for any data point in one experiment. For each time point cumulative shaking time in mentioned in figure. Speed and motion of shaking of most recent shaking is mentioned in brackets. Error bars denote standard deviations.

To see if adding shaking step right after addition of reagent reduces variability or not, we performed experiment similar to figure 23 except with shaking the plate right after adding the reagent this time. Interestingly, %CVs after shaking the plate right after addition of reagent were higher than ones where plate was not shaken ( 8.6 and 10.6 for untreated cells at time points 1 in Figure 23 A and 25 A respectively, and 7.2 and 8 for treated cells at time points 1 in Figure 23 A and 25 A respectively). Secondly, shaking the plate at first time point did not lead to reduction in %CV in at least two subsequent time points as much as seen without shaking plate at first time point (First three time points in untreated cells in 23 A are 8,6.1 and 4.5, whereas in 25 A are 10.5, 9.6 and 8.7). Therefore, we found that the optimal shaking duration to achieve lowest %CVs was 10 minutes, where shaking of the plates was started 10 minutes after addition of the reagent.



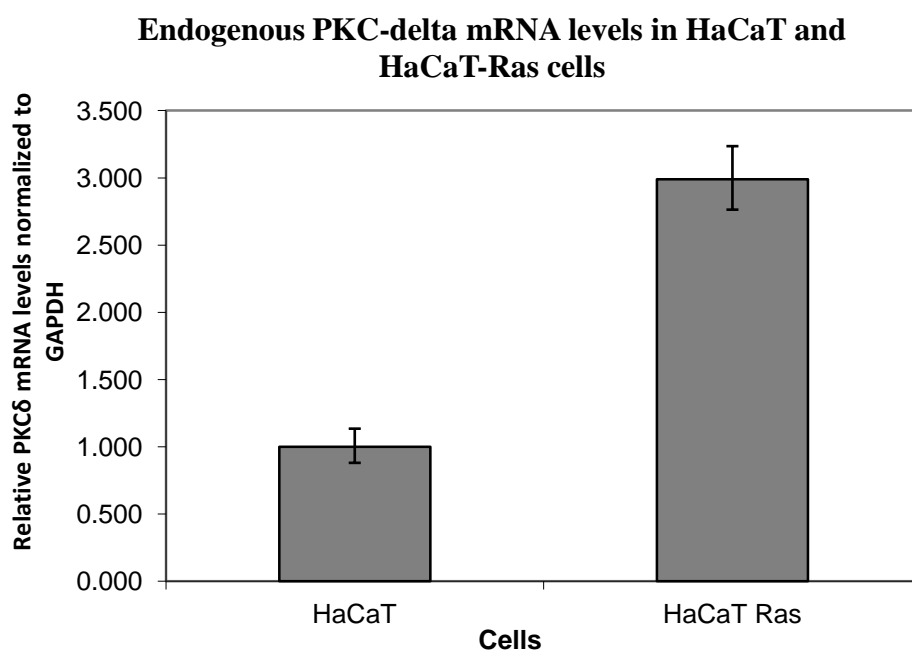
**Figure 25 (A): Shaking plate right after addition of luciferase reagent is not beneficial to reduce variability:** pGL3-hPKC- $\delta$ -4.4 kb transfected HaCaT-Ras cells were plated in 96 well plate. 24 hours after plating, cells were treated with combination of 25  $\mu$ M Bay 11-7085 and 50 nM TPA. 48 hours after drug treatments, luminescence was measured in PHERAstar FS at different time points described in figures. Briefly, for first time point, plate was shaken for 5 minutes right after addition of reagent and luminescence was measured 10 minutes after shaking. Each of subsequent measurements included additional 5 minutes shaking period. (A) shows %CV calculated from (B) luminescence values for all groups (See next page). For each time point cumulative shaking time in mentioned in figure. Speed and motion of shaking of most recent shaking is mentioned in brackets.



**Figure 25 (B): Shaking plate right after addition of luciferase reagent is not beneficial to reduce variability:** pGL3-hPKC- $\delta$ -4.4 kb transfected HaCaT-Ras cells were plated in 96 well plate. 24 hours after plating, cells were treated with combination of 25  $\mu$ M Bay 11-7085 and 50 nM TPA. 48 hours after drug treatments, luminescence was measured in PHERAstar FS at different time points described in figures. Briefly, for first time point, plate was shaken for 5 minutes right after addition of reagent and luminescence was measured 10 minutes after shaking. Each of subsequent measurements included additional 5 minutes shaking period. (B) shows luminescence values for all groups.  $n = 6$  for all data points, where  $n$  = number of replicates for any data point in one experiment. For each time point cumulative shaking time in mentioned in figure. Speed and motion of shaking of most recent shaking is mentioned in brackets. Error bars denote standard deviations.

### PKC- $\delta$ mRNA levels in HaCaT-Ras cells

We were concerned if our HaCaT-Ras cells still had repressed PKC- $\delta$  gene expression because (i) we always observed high basal PKC- $\delta$  reporter activity in transiently transfected HaCaT-Ras cells (Figure 9, 10 A), and (ii) PKC- $\delta$  reporter activity could not be highly induced in these cells even at higher concentrations of PP2 (Figure 16). To answer this question, we measured steady state mRNA levels of PKC- $\delta$  in HaCaT-Ras cells by qRT-PCR and found that they were not lower compared to those in the HaCaT cells (Figure 26), as had been reported previously (17,23). Instead, PKC- $\delta$  mRNA levels were three fold higher in HaCaT-Ras cells than those in HaCaT cells (Figure 26).

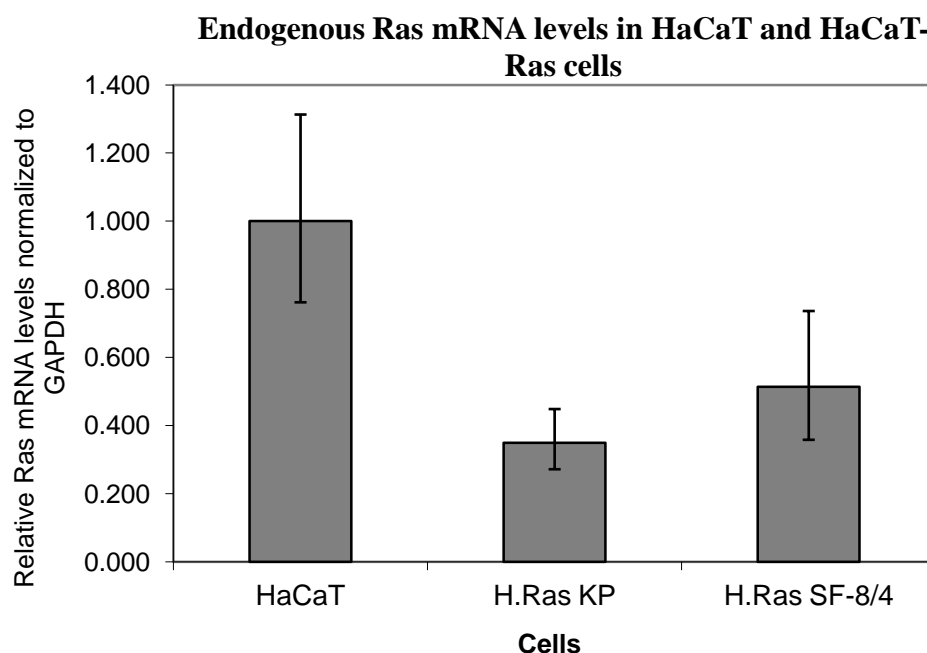


**Figure 26: PKC- $\delta$  mRNA levels are not reduced in HaCaT-Ras cell line:** RNA from both the HaCaT and HaCaT-Ras cells were isolated, and qRT-PCR was performed to check mRNA levels of PKC- $\delta$  after conversion of RNA to cDNA. n=3 for all data points, where n = number of replicates for any data point in one experiment. Error bars denote standard deviations.



### Ras mRNA levels in HaCaT-Ras cells

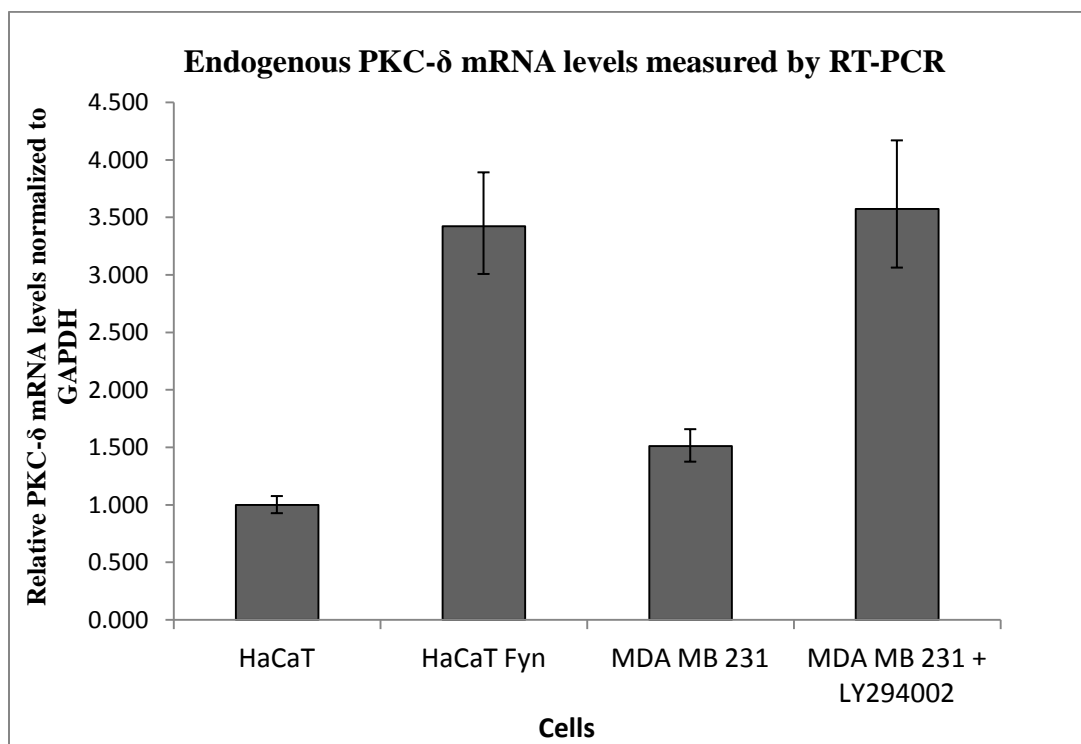
Since Ras is an upstream mediator of our proposed signaling pathway leading to PKC- $\delta$  gene repression in HaCaT-Ras cells (25), we measured mRNA levels of Ras in HaCaT-Ras cell lines to see if they still over-express Ras protein compared to HaCaT cells. Steady state mRNA levels of Ras were not up regulated in two different thaws of HaCaT-Ras cells of our laboratory compared to HaCaT cells (Figure 27), as determined by qRT-PCR.



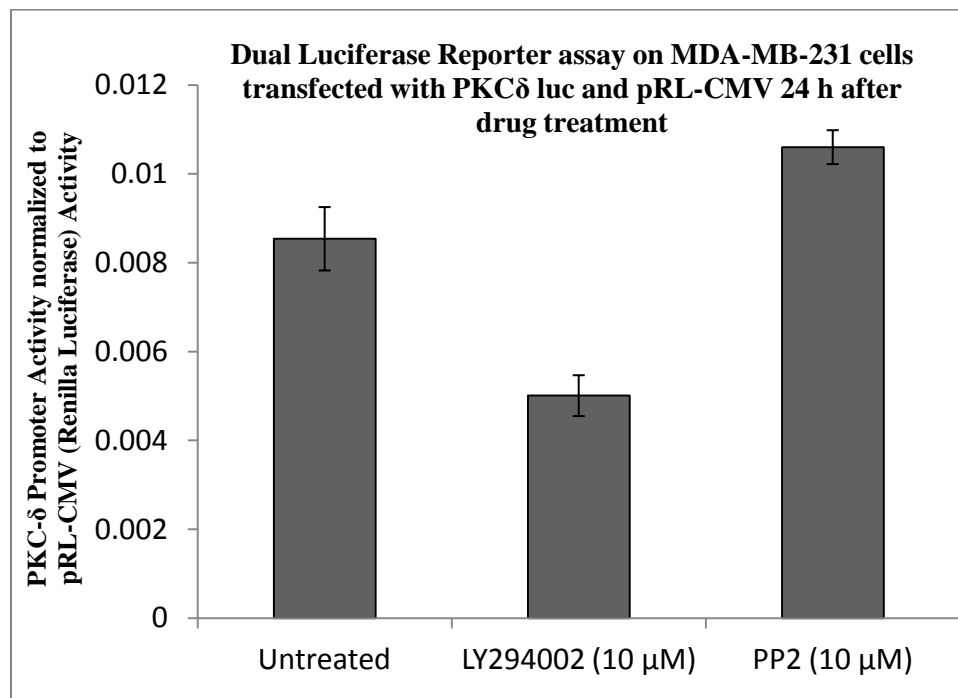
**Figure 27: Ras mRNA levels are reduced in HaCaT-Ras cell line:** RNAs from HaCaT and two different thaws of HaCaT-Ras cells, HaCaT-Ras KP and HaCaT-Ras SF were isolated, and qRT-PCR was performed to check mRNA levels of Ras after conversion of RNA to cDNA.  $n=3$  for all data points, where  $n$  = number of replicates for any data point in one experiment. Error bars denote standard deviations. H.Ras KP = HaCaT-Ras cells cultured by Kushal Prajapati, and H.Ras SF = HaCaT-Ras cells cultured by Sarah Fenton. Both the H.Ras KP and H.Ras SF belonged to the same frozen stock of HaCaT-Ras cells.

### **PKC- $\delta$ mRNA levels in HaCaT-Fyn cells**

Since HaCaT-Ras cells no longer had lower endogenous PKC- $\delta$  levels (Figure 26) and higher endogenous Ras levels, we sought to find out another cell line having reduced PKC- $\delta$  levels that could be used to continue this study. We assessed endogenous PKC- $\delta$  mRNA levels of Fyn transformed HaCaT cells, HaCaT-Fyn as high Fyn activity may lead to reduction in PKC- $\delta$  gene expression (Figure 4). We also checked PKC- $\delta$  mRNA levels of triple negative breast cancer cells, MDA-MB-231 with or without 10  $\mu$ M LY294002 treatment to see if they have PI3K mediated repression of PKC- $\delta$  transcription. Using qRT-PCR, we found that HaCaT-Fyn cells had higher PKC- $\delta$  mRNA levels compared to HaCaT cells (Figure 28). When MDA-MB-231 cells were treated with 10  $\mu$ M LY294002, they showed higher PKC- $\delta$  mRNA levels compared to untreated control (Figure 28). However, this data could not be recapitulated in a dual luciferase assay, wherein we found decrease in PKC- $\delta$  mRNA levels in MDA-MB-231 cells after LY294002 treatment (Figure 29).



**Figure 28: PKC- $\delta$  mRNA levels in HaCaT-Fyn and MDA-MB-231:** HaCaT-Fyn, MDA-MB-231 cells were plated in a way that they were around 60% confluent the next day. On next day, treatment with 10  $\mu$ M LY294002 was started on MDA-MB-231. 24 hours after that RNA from all cells were isolated, and qRT-PCR was performed to check mRNA levels of PKC- $\delta$ .  $n=3$  for all data points, where  $n$  = number of replicates for any data point in one experiment. Error bars denote standard deviations.



**Figure 29: Dual luciferase assay on MDA-MB-231 cells after different drug treatments:** pGL3-hPKC $\delta$ -4.4 and pRL-CMV were co-transfected into MDA-MB-231 cells in relative amounts of 9:1 using Lipofactamine. 24 hours after transfection, cells were treated with indicated concentrations of PP2 and LY294002. Dual luciferase assay was performed 24 hours following drug treatments. Each data point shown in figure is average of three replicates. Error bars denote standard deviation.

## CHAPTER 4

### DISCUSSION

The goal of this study was to develop a stable, high-throughput screening PKC- $\delta$  reporter assay that allows screening of high number of compounds having a potential to induce PKC- $\delta$  gene expression in both academic and industrial settings. To develop a stably transfected cell line, drug selection approach is a commonly utilized by scientists (57, 58). In our case, HaCaT-Ras cells were resistant to most of the selection drugs but the blasticidin. We did not have any mammalian-expression vector carrying blasticidin resistance gene to select the transfected cells using blasticidin. Although we could sub-clone a blasticidin resistance gene in mammalian-expression vector, we proposed an alternative approach of developing a stably transfected cell line to save time. We proposed an approach which involved co-transfection with PKC- $\delta$  reporter plasmid and EGFP, followed by flow-cytometric sorting of EGFP<sup>+</sup> cells to select stably transfected cells. Although the drawback of this approach is that cells are not always under selection pressure to allow elimination of cells which lose plasmids over time, the advantage is that cells stably transfected with EGFP could be easily determined by observing fluorescent cells under microscope. Hereby, we utilized this approach and were successful in generating a HaCaT-Ras cell line stably expressing the pGL3-hPKC $\delta$ -4.4 reporter plasmid.

For selecting the most appropriate clone for assay development, growth, stable transfection and inducibility of reporter activity after treatment with positive control compound PP2 were major criteria. Although we obtained a clone stably transfected with pGL3-hPKC $\delta$ -4.4kb plasmid (Clone 9), inducibility of reporter activity after PP2 treatment was absent in all the clones. The reason for lack of inducibility in reporter in clones may be attributed to the fact that at some point of time HaCaT-Ras cells stopped activating our proposed Ras pathway (Figure 4, 26, 27) leading to PKC- $\delta$  promoter repression.

Searching a good positive control compound which robustly induces PKC- $\delta$  promoter activity was a challenging task as gene regulation of PKC- $\delta$  has not been studied extensively in the past, and so not many compounds increasing PKC- $\delta$  gene expression are known. To find out a positive control that highly induces our 4.4 kb-PKC- $\delta$  promoter activity, we chose specific compounds which would inhibit components of proposed Ras signaling pathway (Figure 4), and compounds which increased PKC- $\delta$  gene expression in other studies. We found the src family kinase inhibitor PP2 to be the best inducer of PKC- $\delta$  promoter activity in HaCaT-Ras cells (Figure 16). The results of NF- $\kappa$ B inhibitor-Bay 11-7085 alone leading to reduction in PKC- $\delta$  promoter activity (Figure 18, column 2, 3) suggested that NF- $\kappa$ B might be a positive regulator of PKC- $\delta$  transcription. This was in congruence with earlier human PKC- $\delta$  promoter studies reported on mouse fibroblasts by Liu *et al.* (25). Interestingly, combination of Bay 11-7085 and PKC activator-TPA induced PKC- $\delta$  promoter activity, as a result of synergy between the two compounds. On the other hand, TNF- $\alpha$ , vitamin D3, estrogen, and insulin did not induce PKC- $\delta$  reporter activity. These findings were not consistent with previous reports (24, 63-65), possibly

because the cell systems used in those studies were different than our cell model system. Substantial efforts were put to optimize the high-throughput assay protocol in PHERAstar FS. For all the single luciferase assays carried out in PHERAstar FS using Steady-Glo Luciferase Assay System, it should be noted that there was no internal control (such as Renilla luciferase) to normalize the data for transfection efficiency like in dual luciferase assays, and it was assumed that transfection efficiencies of pGL3-hPKC $\delta$ -4.4 for all the HaCaT-Ras cells plated in multiple wells were similar. Also in these experiments Steady-Glo Luciferase Assay System was injected into wells using automatic injectors of PHERAstar FS against Promega's protocol recommendation of not using automatic injectors for this reagent to avoid frothing. We used relatively low automatic injection speed in PHERAstar FS, and found no visual evidence of frothing in media on the plate after automatic injection.

The Z value was our yardstick to assess the usability of high-throughput assay developed in this project. We were able to develop an acceptable assay ( $Z=0.19$ ) using PP2 as a positive control in this study, however were not successful in generating excellent assay having Z value 0.5 or higher. To improve quality of the assay, we aimed to increase the fold induction in PKC- $\delta$  promoter activity after drug treatment and reduce variability (%CV) of luminescence. As PP2 treatment caused reduction in number attached HaCaT-Ras cells when observed under the microscope, we thought that it was important to normalize luciferase activity with cell number or viability to correct for the effect of reduced cell viability on induction in reporter activity for any compound in statistical analysis. Initially in this study we proposed to use EGFP fluorescence as an internal control for cell viability, however we could not do so as EGFP fluorescence did not

correlate with the number of attached cells observed under microscope (Figure 20). The probable reason for this phenomenon was that drug compounds had an effect on the transcription of EGFP through interaction with CMV promoter of pEGFP-C1 plasmid transfected into HaCaT-Ras cells. Using Alamar Blue and Cell Titer Fluor Viability assays, we showed that treating HaCaT-Ras cells with compounds such as PP2 reduces their viability (Figures 21, 22). However, both Alamar Blue and Cell Titer Fluor Viability assays interfered with luminescence measurements (Data not shown). Thus multiplexing any suitable cell viability measurement kit in a high throughput luciferase assay in a way that it does not affect luciferase measurements, and health of cells is an important challenge that needs to be addressed in future. In an attempt to reduce variability in luminescence measurements, we started shaking 96 well plates before reading luminescence on PHERAstar FS. As reported in an earlier study (66), shaking the plates before reading luminescence resulted in reduction in %CV in luminescence. We found that the optimal time point and duration of shaking to achieve lowest %CV values in our protocol were 10 minutes after adding the assay reagent and 10 minutes respectively (Figure 23 A).

At some point of time during this study, our HaCaT-Ras cells lost active Ras pathway we proposed earlier and consequentially stopped expressing lower PKC- $\delta$  and higher Ras mRNA levels. It was difficult for us to explain the reason for this occurrence, however one possible reason we postulated was that HaCaT-Ras cells methylated and silenced retroviral-LTR promoter, which drove stable active Ras expression in these cells. As this signaling pathway was pivotal for this study, we could not continue to optimize the assay further until and unless we had a cell system expressing low PKC- $\delta$  promoter activity and



gene expression as a result of active Ras pathway (Figure 4). We hypothesized that Fyn transformed HaCaT cells, HaCaT-Fyn, have lower PKC- $\delta$  mRNA levels compared to those in HaCaT cells as Fyn plays role in proposed Ras pathway downstream of active Ras (Figure 4). However, experimental results we obtained were opposite to our hypothesis, as PKC- $\delta$  mRNA levels were higher in HaCaT-Fyn cells compared to HaCaT cells (Figure 28). Triple negative breast cancer cells MDA-MB-231 harbor endogenous KRAS mutation and were earlier shown to express lower Fyn mRNA levels when treated with LY294002 (67), suggesting PI3K mediated activation of Fyn in these cells. So we premised that MDA-MB-231 cells have active Ras pathway we proposed (Figure 4), and should have higher PKC- $\delta$  promoter activity and gene expression when treated with LY294002. Indeed, we detected higher PKC- $\delta$  mRNA levels (Figure 28), but not PKC- $\delta$  reporter activity (Figure 29) in MDA-MB-231 cells after LY294002 treatment. The probable reason for this contradictory data is that PKC- $\delta$  mRNA levels and promoter activity are not regulated in a similar manner in MDA-MB-231 cells. As a result, we were unsuccessful in finding out any other cell line that could substitute for HaCaT-Ras cells in this study.

In summary, we successfully generated a HaCaT-Ras cell line stably transfected with pGL3-hPKC $\delta$ -4.4. We identified PP2 and combination of Bay 11-7085 and TPA as positive control compounds for developing high throughput PKC- $\delta$  promoter-reporter assay system. We also demonstrated importance of measuring cell viability and proposed Cell Titer Fluor Viability Assay as an assay system of preference to measure cell viability. A detailed protocol for this high throughput assay with specific focus on increasing Z value and convenience of operation in PHERAstar FS was developed in this

project. In future, stable cell system having active Ras pathway (Figure 4) and low PKC- $\delta$  gene expression should be found out to verify findings of this study and continue improving the assay to obtain higher Z values. Human SCC cell line with endogenous HRAS mutation and low PKC- $\delta$  gene expression would be a reasonable substitute cell model. As an alternative approach, if active Ras pathway (Figure 4) could be transiently induced into HaCaT cells by transfection or viral infection of active Ras, a transient cell-based assay to measure PKC- $\delta$  promoter activity could be developed. The tentative design of this transient assay in HaCaT cells comprises transient transfection or infection of active Ras, followed by transient transfection of luciferase reporter plasmids pGL3-hPKC $\delta$ -4.4 and pRL-CMV (Renilla internal control), and measurement of luciferase activities at the last stage. Another possible approach is to induce over-activation of Ras in the HaCaT cells by treating them with EGF (22) and utilize them as a substitute for HaCaT-Ras cells.

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